STUDIES ON NUTRACEUTICAL FUNCTIONS OF SULFUR-CONTAINING COMPOUNDS IN FOODS

（食品由来含硫化合物の生理機能に関する研究）

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LIST OF ABBREVIATIONS

ApoB100	apolipoprotein B100
BSA	bovine serum albumin
CHD	coronary heart disease
DAG	diacylglycerol
DGAT	diacylglycerol acyltransferase
DMEM	dulbecco s modified Eagle medium
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immuno sorbent assay
ER	endoplasmic reticulum
FCS	fetal calf serum
G6PDH	glucose-6-phosphate dehydrogenase
LDL	low density lipoprotein
LPDS	lipoprotein-depleted serum
MTP	microsomal triacylglycerol transfer protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS	phosphate-buffered saline
PAP	phosphatidate phosphohydrolase
TAG	triacylglycerol
TLC	thin layer chromatography
VLDL	very low density lipoprotein

CHAPTER I

INTRODUCTION

INTRODUCTION

The potential therapeutic and health-promoting effects of dietary components are attracting considerable interest, because along with changes in dietary habits and lifestyle, various lifestyle-related diseases, such as hyperlipidemia, obesity and diabetes, are increasing. Onion (*Allium cepa L.*) and garlic (*Allium sativum L.*) have been long used as traditional medicines and are thought to have various effects, including prevention of heart diseases (Augusti, 1996). Among the potential therapeutic application of garlic, the hypolipidemic effects have been reported in studies in human hepatocytes (Gebhardt, 1993), as well as in experimental rats (Kamanna and Chandrasekhara, 1982; Ogawa *et al.*, 1993; Chi *et al.*, 1982) and chickens (Asaf *et al.*, 1983). Adler *et al.*(1997) reported that garlic supplementation significantly decreased both total cholesterol and low density lipoprotein (LDL)-cholesterol in hypercholesterolemia in humans. It has been also reported that oils of onion and garlic extracts inhibit platelet aggregation *in vitro* (Morimitsu *et al*., 1990). Furthermore, the mixtures of onion and garlic exhibited a synergistic effect of inhibition on platelet aggregation compared to each individual one (Morimitsu *et al*, 1992).

On the other hand, epidemiological studies suggest that onion feeding has beneficial effects on the prevention for some diseases (Augusti *et al*., 1975; Causey *et al*., 2000). Some *in vitro* studies indicated that onions have been reported to exhibit anti-bacterial (Al-Delaimy and Ali, 1970) and anti-platelet aggregative (Harata and Matsushita, 1996). Recently, Chen *et al.* (1999) investigated whether onion extracts could alter

vascular responses *in vitro* in the thoracic aorta of SD-rats because onion has been consumed for the prevention of cardiovascular disorders in Asian countries. They demonstrated that Welsh onion extracts caused vasodilation on precontracted vessel rings and concluded that boiled onion extract could stimulate the release of an endothelium-derived contracting factor, which was assumed to be thromboxane A2. Helen *et al.* (2000) reported that onion oil was an effective antioxidant against the oxidative damage caused by nicotine. Sheela *et al.* (1995) studied the anti-diabetic effects of onion and garlic sulfoxide amino acids in rats, and they found that on oral administration of onion and garlic sulfoxide amino acids to alloxan-diabetic rats was ameliorated as comparable to rats treated with insulin. However, it is still scarce of the information on the effect of onion on physiological functions. In addition, little is known about components of onion responsible for the biological activities. To answer these questions, we conducted to study on the effects of onion on lipid synthesis and lipoprotein assembly. It is known that lipid and lipoprotein metabolism were altered under the many physiological conditions and by several mechanisms. Typical metabolic disorder is a hyperlipidemia, which is related to the cause of many lifestyle-related diseases.

Hyperlipidemia is a syndrome characterized by excessive accumulation of lipids in the bloodstream (Loscalzo and Sniderman, 1993). Apolipoprotein B100 (apoB100)-containing lipoproteins are assembled in the liver. ApoB100-containing lipoproteins are synthesized with supply of TAG, phospholipid, cholesterol, and apoB100. Recently, it is also confirmed that microsomal TAG transfer protein (MTP) is necessary to assemble these lipoproteins (Wetterau *et al*., 1992). ApoB100 is an essential protein component

of very low density lipoprotein (VLDL) and LDL, and is required for the intracellular assembly and secretion of these lipoproteins (Fig.1) (Scott *et al*., 1994). However, the overproduction of VLDL in the liver resulted in a hyperlipidemia. Triacylglycerol (TAG) biosynthesis is catalyzed by three enzymes, glycerol-3-phosphate acyltransferase, phosphatidate phosphohydrolase, and diacylglycerol acyltransferase (DGAT) in the liver (Haggsman *et al*., 1981; Owen *et al.*, 1997) (Fig.2). Phosphatidate phosphohydrolase and DGAT are postulated to be key enzymes for TAG biosynthesis. They are located in endoplasmic reticulum as active forms (Brindley *et al*., 1984; Yanagita *et al*., 1987).

ApoB100, a 556 kDa hydrophobic protein, is synthesized in the endoplasmic reticulum (ER) and shows LDL-receptor binding activity (Davis, 1999; Twisk *et al*., 2000). After synthesis, it is stabilized through binding with lipids, and then is secreted as apoB100-containing lipoproteins into the bloodstream. If sufficient lipid is not available, hydrophobic apoB100 is unstable and undergoes degradation *via* the ubiquitin-proteasome pathway (Fig. 3)(Fisher *et al*., 1997). Therefore, the control of apoB100 expression in the cells is considered to be post-transcriptional (Gordon *et al*., 1996; Yanagita *et al*., 1999-b). It is known that VLDL plays an important role to transport TAG as well as cholesterol to the tissues.

MTP catalyzes the transfer of TAG, cholesterol ester, and phosphatidylcholine between membranes, which is formed in the lumen of microsomes isolated from the liver and intestinal mucosa (Jamil *et al*., 1995; Benoist and Grand-Perret, 1997). MTP is a heterodimer consisting of two subunits of molecular mass of 58 kDa and 97 kDa. The 58 kDa component of MTP has been identified as the multifunctional protein, protein

disulfide isomerase (Wetterau *et al*., 1990). The large 97 kDa possesses the lipid transfer activity. The large subunit of MTP was not detectable in subjects with the disease abetalipoproteinemia, a rare human genetic disease characterized by a defect in VLDL and chylomicron assembly (Gregg and Wetterau, 1994; Wetterau *et al*., 1992). It is known that there are distinct relationships between the onset of coronary heart disease (CHD) and higher levels of serum lipids and apoB100 (Cullen *et al*., 1997; Davis and Vance, 1996).

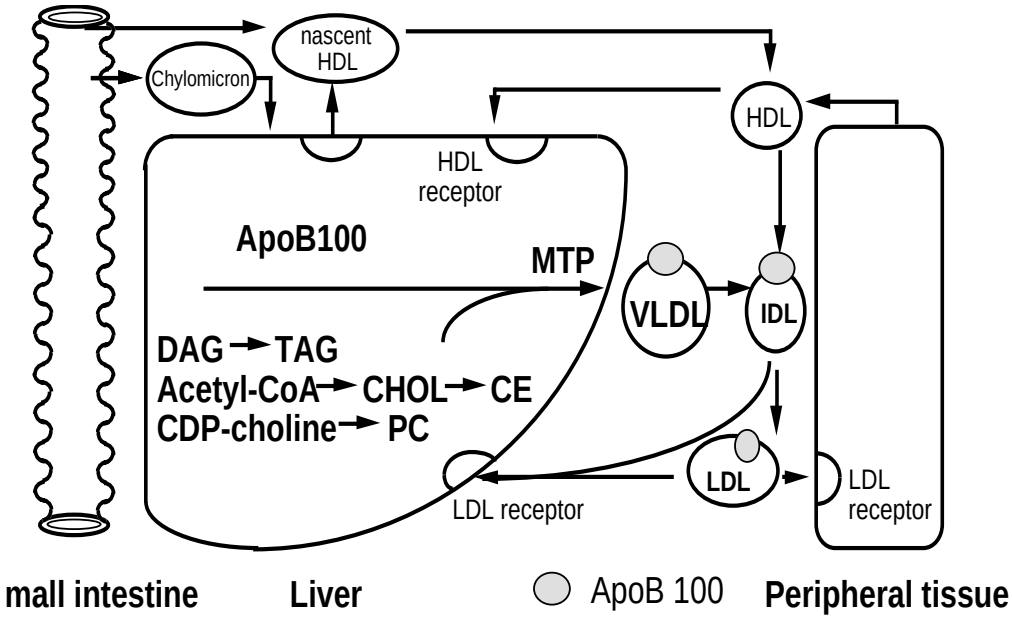
In the preliminary study, it was observed that dietary onion powder reduced serum TAG levels in rats, suggesting the hypolipidemic activity of onion. *Allium* species such as onion and garlic contain relatively abundant amounts of sulfur-containing compounds and they exert for the characteristic odor and taste (Brock, 1995; Matsukura, 1994). Biological actions of onion and garlic are, in part, ascribed to sulfur-containing compounds. Thus, to extend the therapeutic application and to identify the components responsible for the nutraceutical actions, we focused our study to investigate the effects of main sulfur-containing compounds, cycloalliin, which is the derivatives of cysteine and methionine, and sulfoxide compounds found in onion on the lipid and lipoprotein metabolism. In Chapter II, the effects of onion extract on the growth performance and lipid metabolism were studied. Cycloalliin, 3-(S)-methyl-1,4-thiazane-5-(R)-carboxylic acid-(S)-oxide, is the most abundant sulfur-containing imino acid in onion and garlic. It has the great merit of being almost flavorless and stable (Table. 1-1)(Ueda *et al*., 1994). In Chapter III, we studied the effects of cycloalliin on lipid metabolism and apoB100 secretion by using experimental rats and human hepatoma cell line, HepG2.

Table 1-1. Composition of major sulfur-containing compounds in onion

	(mg/100 g onion bulb)	
	Raw	Heated
Cycloalliin	28.2	104.3
S-Methyl-cysteine sulfoxide	46.5	41.5
S-Propenyl-L-cysteine sulfoxide	82.3	8.9
γ-Glutamyl-S-propenyl-L-cysteine sulfoxide	69.3	2.4
S-(2-Carboxypropyl) glutathione	12.5	-
Glutathione	0.4	3.0
Cysteine	13.7	6.5
Methionine	11.5	0.6
Other compounds	trace	trace

(Ueda *et al*., 1994)

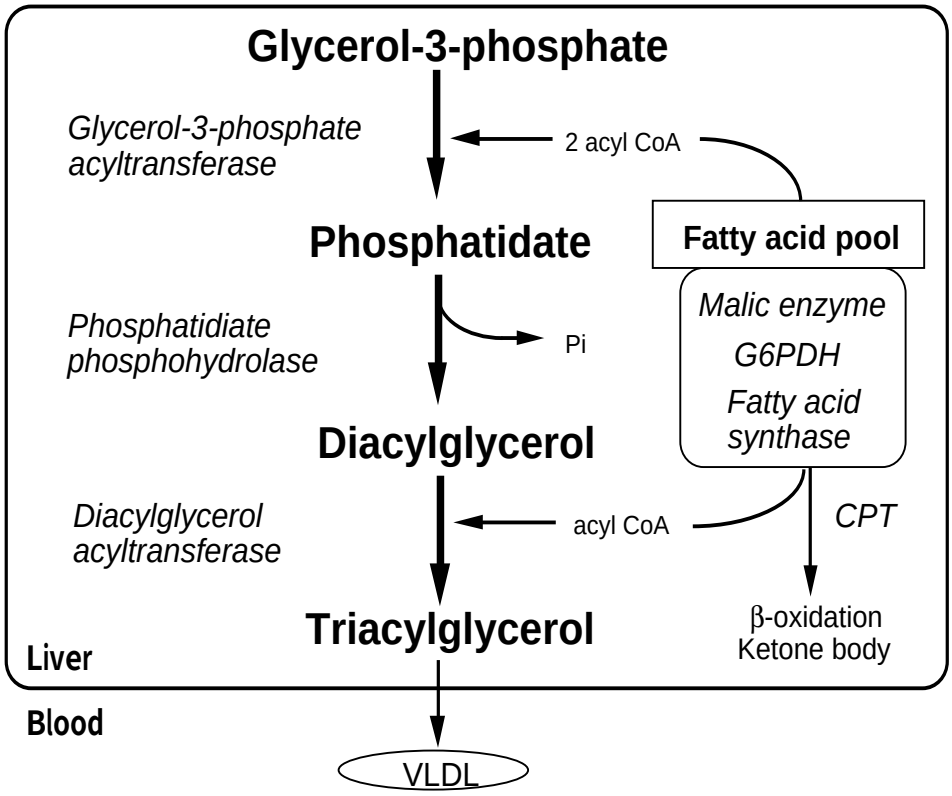
In Chapter IV, we evaluated the effects of S-methyl cysteine, S-ethyl cysteine, S-propyl cysteine, propyl-cysteine sulfoxide, DL-methionine sulfoxide, S-carboxymethyl cysteine, and S-carboxyethyl cysteine from onion on lipid and apoB100-containing lipoprotein metabolism in HepG2 cells.



*ApoB 100 : Apolipoprotein B100, DAG : Diacylglycerol, TAG : Triacylglycerol
CHOL : Cholesterol, CE : Cholesterol ester, PC : Phosphatidylcholine
HDL : High density lipoprotein, LDL : Low density lipoprotein,
IDL : Intermediate density lipoprotein, VLDL : Very low density lipoprotein
MTP : Microsomal TAG transfer protein

(Scott *et al*., 1994, Wetterau *et al*., 1992)

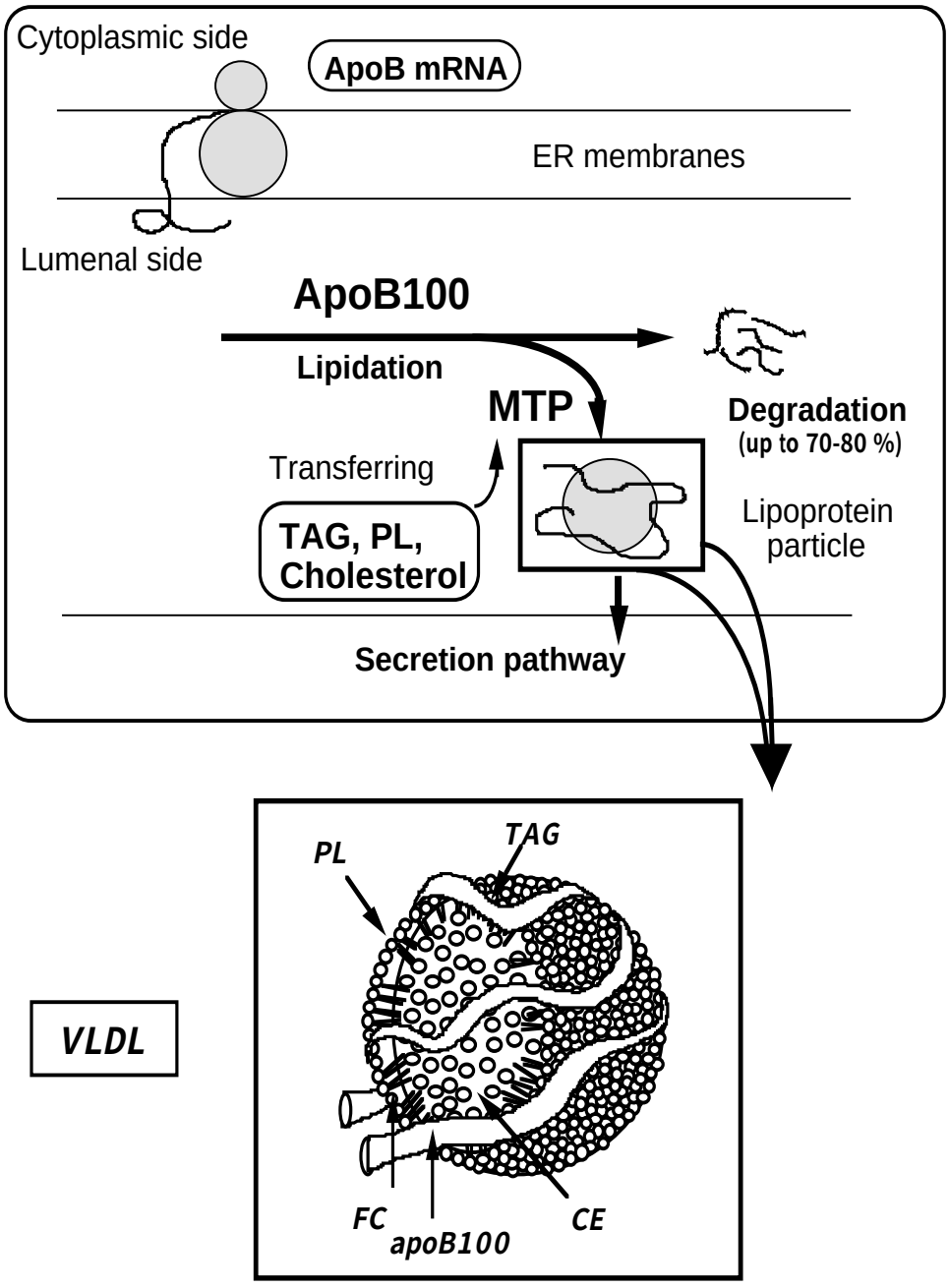
Fig.1. Lipid and lipoprotein metabolism



* **G6PDH**: Glucose-6-phosphate dehydrogenase, **CPT:** Carnitine palmitoyltransferase

(Haggsman *et al*., 1981; Owen *et al*., 1997)

Fig.2. Biosynthesis of triacylglycerol in the liver



(Fisher *et al*., 1997; Gordon *et al*., 1996)

Fig.3. Assembly and secretion of apoB100-containing lipoproteins in the liver

CHAPTER II

Effects of onion extract on body weight, tissue weight, and lipid metabolism in rats

Part 1. Effects of onion extract on body weight, tissue weight, and lipid metabolism in rats

1. INTRODUCTION

Allium species such as onion and garlic has been used as medicine for centuries, but there was little scientific support of its therapeutic properties until recently. The lipid lowering effect of garlic has been reported in several earlier trials (Augusti, 1996; Block, 1992). Furthermore, garlic has been reported to have other medicinal properties including a propensity to inhibit platelet aggregation and reduce cancer risk (Morimitsu *et al*., 1992; Babu and Srinivasan, 1997).

However, little is known whether onion and associated sulfur-components have medicinal properties similar to garlic. The present study was undertaken to know whether supplementation of onion extract to the diet affect body weight gain, tissue weights, and lipid metabolism in experimental rats.

2. MATERIALS AND METHODS

2.1 Experimental material and reagents

Commercial kits for measuring the concentrations of total cholesterol, glucose, TAG, phospholipid and free fatty acid in serum were purchased from Wako Pure Chemical Ind. (Osaka, Japan). All other chemicals and reagents were of special grade available.

2.2 Preparation of onion extract powder

Onion extract used was prepared as the procedure reported by Anno (2000). In brief, onions were blanched in water bath at 80 °C for 1 h, and then homogenated by using a Warning blender. After centrifugation at 8,000 rpm for 30 min, the resulting supernatant was treated with cellulase (Cellulase A, Amono Seiyaku Co., Ltd., Osaka, Japan) and glutaminase (Daiwa C-100, Daiwa Kagaku Co., Ltd., Osaka, Japan). The samples were heated at 90 °C for 60 min, spray-dried, and used as onion extract. The cycloalliin content of the onion extract was 0.55 g per 100 g, which is approximately 5-fold higher concentration than that of the corresponding raw onion.

2.3 Experimental animals

Four-week-aged male SD-strain rats (125-135 g) were obtained from Kyudo (Tosu, Japan) and maintained in an individual stainless-meshed cage on a temperature-controlled room (24 °C) with a 12 h light/dark cycle. They were divided into three groups with equal body weight (6 rats in each group) after rearing with a stock pellet chow (CE-2, CLEA, Tokyo, Japan) for 7 days. Control group was given the semipurified atherogenic diet containing 0.5 % cholesterol and 0.125 % sodium cholate. The remaining two groups were fed the same diets supplemented with 0.4 % and 1.2 % of onion extract. The composition of experimental diets is presented in Table 2-1. The animals had free access to the diet and water. Food intake was recorded everyday, and body weight was recorded every other day during the experimental period. After 2 weeks, they were fasted for 5 h, and then blood was collected from *vena cava* and the liver was excised under diethyl ether-anesthesia. The study was approved by Saga University Institutional Animal Care and

Use Committee.

2.4 Lipid analyses

Lipids were extracted and purified by the method of Folch *et al*. (1957). The concentrations of hepatic TAG and cholesterol were measured by the methods of Fletcher (1968) and Sperry and Webb (1950), respectively. The hepatic phospholipid was quantified by phosphorus content as reported previously, and multiplied by 25 to extrapolate the phospholipid molecules (Barlett, 1959). Serum was separated by centrifuging the blood at 3,000 rpm for 15 min. The concentrations of total cholesterol, HDL-cholesterol, TAG, and phospholipid in the serum were measured enzymatically with commercial kits supplied by Wako Pure Chemical Ind. (Osaka, Japan).

2.5 Preparation of liver subcellular fractions

Liver homogenates and subcellular fractions were prepared as described by Yanagita *et al*.(1987). In brief, a piece of liver was homogenized in 4 volume of ice-cold 0.25 M sucrose homogenate solution containing 1 mM EDTA, 0.2 mM dithiothreitol, and 10 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 1,000×g for 10 min to sediment nuclei and cell debris, and the supernatant was then centrifuged at 20,000×g for 20 min at 4 °C to sediment mitochondrial fraction. The resulting supernatant was further centrifuged at 105,000×g for 45 min at 4 °C to sediment microsomes; the supernatant was used as the cytosolic fraction. Microsomal pellet was resuspended in a small volume of the homogenate solution described above. These fractions were stored at -80 °C until assay. Protein concentration was measured by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

2.6 Assays of enzyme activities

Glucose-6-phsphate dehydrogenase (G6PDH, EC 1.1.1.49); The activity of G6PDH was measured as described by Zakim and Donal (1973). The reaction solution contained 0.32 M Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 66 mM glucose-6-phosphate, 24 mM $NADP^+$, and 1 unit of 6-phosphogluconate dehydrogenase. The reaction solution was preincubated at 27 °C, and the reaction was initiated by adding the microsome fraction (250 μg protein) in a final assay volume of 3.0 mL at 27 °C for 2 min. The absorbance was determined at 340 nm. G6PDH activity was expressed as nanomole of enzyme capable of producing NADPH/min/mg protein.

Malic enzyme (EC 1.1.1.40); The activity of malic enzyme was determined as reported by Ochoa *et al.* (1948). The reaction solution contained 0.4 M triethanolamine (pH 7.4), 30 mM malic acid, 0.12 M $MnCl_2$, and 3.4 mM $NADP^+$. The reaction solution was preincubated at 27 °C, and the reaction was initiated by adding the microsome fraction (250 μg protein) in a final assay volume of 3.0 mL at 27 °C for 2 min. The absorbance was determined at 340 nm. Malic enzyme activity was expressed as nanomole of enzyme capable of producing NADPH/min/mg protein.

Phosphatidate phosphohydrolase (PAP, EC 3.1.3.4); The activity of PAP was assayed by the method of Possmayer and Walton with a slight modification (Walton, 1985). The reaction mixtures contained 0.05 M Tris-HCl (pH 7.0), 1 mM L-α-phosphatidate, and 1 mM phosphatidylcholine liposomes suspended in 1.55 M sodium chloride, in the presence of 1.25 mM magnesium sulfate, and 50 to 100 μg of liver enzyme protein in a final assay volume of 0.2 mL. The mixture was incubated for 15 min at 37 °C and

reaction was terminated by the addition of 0.8 mL of a solution containing 0.13 % sodium dodecyl sulfate, 1.25 % ascorbic acid, 0.32 % ammonium molybdate-4H_2O, and 0.75 N H_2SO_4. The liberated inorganic phosphate was measured. The phosphomolybdate color was developed at 45 °C for 20 min and the absorbance was measured at 820 nm. Non enzymatic phosphate release was determined by inactivating the enzymes by boiling for 1 min without substrate. The enzyme activity expressed as nanomole in one minute per mg protein.

Diacylglycerol acyltransferase (DGAT, EC 2.3.1.20); The activity of DGAT was measured as described by Coleman and Cao (1976). In brief, the reaction mixture contained 350 µL of 175 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 30 mM [1-^{14}C] palmitoyl-CoA (specific activity 2.11 MBq /µM), 1mg/mL fatty acid free bovine serum albumin, and 50 µL of 125 mM 1,2-diacylglycerol dissolved in absolute ethanol were employed. The reaction was initiated by adding 100 µL of liver microsome or adipose tissue homogenate (20/50 µg of protein) and blending on a vortex mixer. The reaction was terminated after 10 minutes by adding 3.75 mL of 2-propanol/heptane/H_2O (80:20:1, v/v/v). After 5 min, 2.5 mL of heptane and 1.25 mL of water were added to the reaction mixture and the tubes were blended on a vortex mixer and centrifuged at 3000 rpm for 5 min. One mL of solution from the heptane layer was transferred into scintillation counting vial. Radioactivity was measured using a liquid scintillation counter (Wallic system 1410, Pharmacia, Uppsala, Sweden).

2.7 Statistical analyses

Each value is presented as means ± SE. Data were analyzed by one-way

ANOVA, all differences being inspected by Duncan s new multiple-range test (Duncan, 1955). A difference was considered significant at $p < 0.05$.

3. RESULTS

3.1 Effects of dietary onion extract on body weight gain, food intake, and liver and white adipose tissue weights

Body weight gain, food intake and tissues weights in rats fed the cholesterol-enriched atherogenic diets supplemented with or without onion extract (0.4 and 1.2 %) are shown in Table 2-2. There was no difference in final body weight and food intake among the groups. However, liver weights were decreased significantly in rats fed both diets supplemented with onion extract.

Table 2-3 shows the peritoneal and epididymal adipose tissue weights after administering onion diet. Adipose tissue weights tended to reduce by administering onion extract and lowered significantly at 0.4 % onion group.

3.2 Effects of dietary onion extract on concentrations of serum lipids and glucose

TAG concentration in the serum was lower dose-dependent manner in both onion diets compared to the control diet. The concentrations of cholesterol, phospholipid and free fatty acids in the serum were comparable among the groups, although 0.4 % onion group tended to reduce the levels compared to the other two groups. The serum concentration of glucose was not altered by the supplementation of onion extract (Table 2-4).

3.3 Effects of dietary onion extract on the concentrations of liver lipids

There were no significant differences in the concentrations of hepatic TAG, phospholipid, and cholesterol, although hepatic TAG content per gram tissues tended to higher in both onion groups (Table 2-5). This may be in part associated with the decrease in liver weight or the decrease in serum TAG secretion from the liver in onion-fed rats.

3.4 Effects of dietary onion extract on the enzyme activities involved in fatty acid and TAG syntheses

Hepatic G6PDH and PAP activities were comparable among the three groups. Hepatic malic enzyme activity was comparable between the control and 1.2 % onion groups, but lowered in the 0.4 % onion group compared to the control group. DGAT activity is higher in the 1.2 % onion group compared to the other groups (Table 2-6).

4. DISCUSSION

We demonstrated in the present study that onion extract had hypotriglyceridemic activity and anti-obesity property in the animals fed the atherogenic diets. This is in agreement with the result of Babu and Srinivasan who reported that onion alleviated hypertriglyceridemia in experimental diabetic rats, although they did not found any effect of onion on normolipidemic rats (1997).

In general, the concentration of TAG in the serum is regulated by the rate of TAG synthesis and fatty acid oxidation, and VLDL assembly in the liver and peripheral lipoprotein lipase activity which catalyzes the hydrolysis of TAG to fatty acid and glycerol

(Schulz, 1991). To understand the underlying mechanisms by which onion reduces serum TAG level, hepatic TAG content and enzyme activities involved in TAG and fatty acid syntheses were measured. However, we found no change in hepatic TAG level after administering onion. In addition, we did not find any consistent effects of onion extract on malic enzyme and G6PDH, both of which catalyze NADPH formation for fatty acid synthesis. Furthermore, there was no significant consistent change in hepatic PAP and DGAT activities by administering onion (Table 2-6). Taken together, it is concluded that supplementation of onion extract to the atherogenic diet lower serum TAG concentration without affecting TAG synthesis in the liver.

It is postulated that dietary onion and garlic have hypocholesterolemic effects (Sheela *et al*., 1995), however, no effects on cholesterol concentration in the serum and liver were observed. In addition, there was no difference in serum glucose and free fatty acid levels. It suggests that onion extract used in this experiment might be no influence on diabetic disorder. To further clarify this point, measurement of serum insulin level should be required.

Dietary onion leads to reduce serum TAG level, in spite of the no significant change in hepatic lipid content and lipid synthesis. We hypothesize that onion components might inhibit the assembly and secretion of apoB100-containing lipoproteins in the liver. It is believed that assembly of apoB100-containing lipoprotein is regulated by lipid availability, apoB100 metabolism, and MTP activity. Recent report indicates that the inhibition of hepatic MTP reduces the serum lipid levels and induces the regression of atherosclerosis (Wetterau *et al*., 1998). In agreement with this, we have shown that the

administering onion component causes to reduce hepatic MTP activity and it may associated with the lower serum TAG concentration in rats in the following Chapter. Lipoprotein lipase activity was not determined in the present study.

It is also interesting note that dietary onion results to decrease liver weight and white adipose tissues, although the underlying mechanisms are not apparent at present time. As obesity is a key disease of many common diseases, onion is thought to be a beneficial food for health. The onion extract used in this study was prepared by the treatments with blanching and then with cellulase and glutaminase. This procedure is reported to enrich five-fold the cycloalliin content in the treated onion by Anno (2000).

In conclusion, dietary onion reduces serum TAG concentration and adipose tissue weight in rats fed the atherogenic diet.

Table 2-1. Composition of experimental diet.

Ingredients	Control	Onion extract	
	0 %	0.4 %	1.2%
Casein	20.0	20.0	20.0
DL-Methionine	0.3	0.3	0.3
Vitamin mixture	1.0	1.0	1.0
Mineral mixture	4.0	4.0	4.0
Cellulose	2.0	2.0	2.0
α-Corn starch	15.0	15.0	15.0
Choline bitartrate	0.2	0.2	0.2
Cholesterol	0.5	0.5	0.5
Sodium cholate	0.125	0.125	0.125
Fat (Beef tallow	5.0	5.0	5.0
Fat (Corn oil	5.0	5.0	5.0
Sucrose	43.875	43.475	43.675
Dextrin	3.0	3.0	3.0
Onion extract	0.0	0.4	1.2

Table 2-2. Effect of dietary onion extract on the growth performance and the food intake.

Groups	Control	Onion extract	
	0 %	0.1 %	0.3 %
Body weight (g)			
Initial	150.7±2.9	150.9±2.8	149.8±3.1
Final	261.4±6.9	259.6±4.8	254.0±5.9
Liver weight			
total (g)	15.8±0.6[a]	13.9±0.4[b]	13.9±0.5[b]
(g/100g B.W.)	6.1±0.2[a]	5.4±0.2[b]	5.5±0.2[b]
Food intake			
(g/day)	19.9±0.7	19.2±0.4	19.2±0.4

Four-week-aged SD male rats fed the semipurified diets supplemented without (control) or with onion extract at the levels of either 0.4 % or 1.2 % for two weeks. Values are expressed as mean ± SE of six in each groups. Values with different letters are significantly different at $p < 0.05$.

Table 2-3. Effect of dietary onion extract on adipose tissue weight.

Groups	Control	Onion extract	
	0 %	0.4 %	1.2 %
		(g)	
Perirenal	1.62±0.20[a]	1.07±0.14[b]	1.21±0.14[a]
Epididymal	1.81±0.27	1.40±0.08	1.42±0.14

Four-week-aged SD male rats fed the semipurified diets supplemented without (control) or with onion extract at the levels of either 0.4 % or 1.2 % for two weeks. Values are expressed as mean ± SE of six in each groups. Values with different letters are significantly different at $p < 0.05$.

Table 2-4. Effect of dietary onion extract on serum metabolites.

Groups	Control	Onion extract	
	0 %	0.4 %	1.2 %
		(mg/dL)	
Triacylglycerol	237.6±30.0[a]	195.5±31.3[a]	147.6±23.5[b]
Phospholipid	180.1±14.1	169.9±11.3	183.2±16.6
Cholesterol	139.0±21.6	116.8±10.3	141.3±11.3
Glucose	137.1±12.8	138.2±6.7	129.0±11.0
		(μEq/L)	
Free Fatty Acid	663.2±48.3	627.3±61.3	661.8±40.3

Four-week-aged SD male rats fed the semipurified diets supplemented without (control) or with onion extract at the levels of either 0.4 % or 1.2 % for two weeks. Values are expressed as mean ± SE of six in each groups. Values with different letters are significantly different at $p < 0.05$.

Table 2-5. Effect of dietary onion extract on the hepatic lipid levels.

Groups	Control	Onion extract	
	0 %	0.4 %	1.2 %
		(mg/g liver)	
Triacylglycerol	71.2±2.4	75.4±6.4	75.3±11.5
Phospholipid	28.4±1.9	29.4±1.1	29.4±1.1
Cholesterol	37.0±1.5	39.0±3.6	40.4±2.1

Four-week-aged SD male rats fed the semipurified diets supplemented without (control) or with onion extract at the levels of either 0.4 % or 1.2 % for two weeks. Values are expressed as mean ± SE of six in each groups.

*Abbreviation: PAP: phosphatidate phosphohydrolase, G6PDH: glucose-6-phosphate dehydrogenase, ME: malic enzyme, DGAT: diacylglycerol acyltransferase.

Table 2-6. Effect of dietary onion extract on the activities of hepatic enzymes.

Groups	Control	Onion extract	
	0 %	0.4 %	1.2 %
	(nmol/min/mg protein)		
G6PDH	116.9±4.8	104.7±4.9	106.3±6.0
PAP	9.5±0.6	9.0±1.0	9.7±0.4
ME	40.6± 3.2[a]	33.7±2.0[b]	37.9±4.9[a]
DGAT	2.9±0.3[a]	2.8±0.3[a]	4.1±0.4[b]

Four-week-aged SD male rats fed the semipurified diets supplemented without (control) or with onion extract at the levels of either 0.4 % or 1.2 % for two weeks. Values are expressed as mean ± SE of six in each groups. Values with different letters are significantly different at $p < 0.05$.

CHAPTER III

Effects of cycloalliin on lipid metabolism in rats and in HepG2 cells

Part 1. Effects of dietary cycloalliin on lipid metabolism and the related enzyme activities in rats fed cholesterol-enriched diets

1. INTRODUCTION

We demonstrated in Chapter II that dietary onion extract had a hypotriglyceridemic effect, accompanying with decreases in adipose tissue weights in the rat. It is well known that onion and garlic contain a variety of sulfur-containing compounds (Block, 1992). Cycloalliin is a main sulfur-containing imino compound with a cyclic structure and its content is markedly increased in onion after cooking, which accounts for about 50 % of all sulfur-containing compounds (Ueda *et al*., 1994). It is suggested that by heating-treatment, S-propenyl-cysteine sulfoxide and other sulfur-containing compounds in onion appear to form a stable cyclic structure, cycloalliin (Fig. 4). It has been reported that cycloalliin promotes a fibrinolytic activity in the blood (Agarwal *et al*., 1977). However, little is known about other physiological activities of this compound. In this part, we studied the effects of dietary cycloalliin on liver and serum lipid concentrations and on enzyme activities related to lipid metabolism in rats fed cholesterol-enriched diets.

2. MATERIALS AND METHODS

2.1 Experimental material and reagents

Cycloalliin (cycloalliin hydrochloride monohydrate) was supplied by Nippon Shinyaku (Kyoto, Japan). All other chemicals and reagents were of special grade available.

2.2 Experimental animals and diets

Four-week-aged male SD-strain rats (125-135 g) were obtained from Kyudo (Tosu, Japan) and maintained in an individual stainless-meshed cage on a temperature-controlled room (24 °C) with a 12 h light/dark cycle. They were divided into three groups with equal body weight (6 rats in each group) after rearing with a stock pellet chow (CE-2, CLEA, Tokyo, Japan) for 7 days. Control group was given the semipurified atherogenic diet that contains 0.5 % cholesterol and 0.125 % sodium cholate. The remaining two groups were fed the cholesterol-enriched diets supplemented with either 0.1 % or 0.3 % of cycloalliin. The composition of experimental diets is presented in Table 3-1-1. The animals had free access to the diet and water. Food intake was recorded everyday, and body weight was recorded every other day during the experimental period. After 2 weeks, they were fasted for 5 h, and then blood was collected from *vena cava* and the liver was excised under diethyl ether-anesthesia. The study was approved by Saga University Institutional Animal Care and Use Committee.

2.3 Preparation of liver subcellular fractions.

Liver homogenates and subcellular fractions were prepared as described in Chapter II.

2.4 Lipid analyses

Serum and hepatic lipids were measured as described in Chapter II.

2.5 Assays of TAG synthetic enzymes in the liver

The activities of phosphatidate phosphohydrolase (PAP, EC 3.1.3.4) , glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), and malic enzyme (EC 1.1.1.40) were determined as described in Chapter II.

2.6 Assay of MTP activity

Microsomal TAG transfer protein (MTP) activity was determined by measuring the rate of transfer of radiolabeled TAG between two populations of unilamellar vesicles by the method of Wetterau *et al*. (1986), with a slight modification. In brief, assay buffer was prepared in 15 mM Tris-HCl (pH 7.4), 35 mM NaCl, 0.02 % sodium azide, and 1 mM EDTA (designated as the assay buffer) by bath sonication in a screwcapped tube under the nitrogen gas. Donor vesicle contained 35 nM of phosphatidylcholine (PC), 0.175 nM of TAG, 18.5 kBq [^{14}C]triolein, and 0.1 % butyrated hydroxytoluene. Acceptor vesicle contained 175 nM of PC, 0.875 nM of TAG, 8.8 nM of cardiolipin, and 0.1 % butyrated hydroxytoluene. The final reaction mixture in a total volume of 1.0 mL contained 20 μL of donor vesicle, 100 μL of acceptor vesicle, 100 μL of 5 % BSA, microsomal sample (protein content: 50 μg/100 μL), and assay buffer. The reaction was terminated by adding 0.5 mL DEAE-cellulose in 1 volume of assay buffer. The mixture was agitated for 1 min, and then DEAE-cellulose, which adsorbed acceptor vesicle, was sedimented by centrifugation at 3000 rpm for 3 min at room temperature. The radioactivity in an aliquot of the supernatant was determined. Transfer activity was calculated as follows: percent

of transfer activity = dpm ([blank donor vesicle fraction] minus [sample donor vesicle fraction])/dpm (blank donor vesicle fraction)×100.

2.7 Statistical analyses

Each value is presented as means ± SE. Data were analyzed by one-way ANOVA, all differences being inspected by Duncan s new multiple-range test (Duncan, 1955). A difference was considered significant at $p < 0.05$.

3. RESULTS

3.1 Effects of cycloalliin on body weight, food intake, and liver weight

When rats were fed high cholesterol diets supplemented with cycloalliin at 0.1 % and 0.3 % levels for 2 weeks, there were no significant differences in body weight gain and food intake among the groups, as shown in Table 3-1-2. In addition, supplementation of 0.1 % and 0.3 % cycloalliin to the high cholesterol diet did not cause untoward effect on liver weight (Table 3-1-2).

3.2 Effects of cycloalliin on serum and liver lipid levels in rats

The supplementation of cycloalliin at the levels of 0.1 % and 0.3 % to the cholesterol-enriched diets as compared to those without cycloalliin caused 39 % and 44 % reductions in the concentration of serum TAG, respectively (Fig. 5), indicating a dose-dependent reduction of serum TAG levels by cycloalliin. In contrast, cycloalliin had no effects on the concentrations of total- and HDL-cholesterol in the serum, although the concentration of cholesterol ester in the serum showed slight tendency to decrease (Fig.

6). Dietary cycloalliin exerted no significant effects on hepatic concentrations of TAG, cholesterol and phospholipids, although there was a slight tendency to increase in TAG and phospholipid contents (Table 3-1-3).

3.3 Effect of dietary cycloalliin on hepatic activities of PAP, malic enzyme, and G6PDH

Figure 7 shows hepatic enzyme activities regarding TAG and fatty acid syntheses. Neither the activity of microsomal PAP nor that of malic enzyme and G6PDH, NADPH-generating enzyme, remained changed by feeding of 0.1 % and 0.3 % level of cycloalliin in the diet, respectively (Fig. 7).

3.4 Effect of dietary cycloalliin MTP activity

As MTP is required for apoB-containing lipoprotein assembly and secretion, the effect of cycloalliin on hepatic MTP activity was measured in rats fed on cholesterol-enriched diets. As shown in Fig. 8, the MTP activity in rats fed cycloalliin was significantly lower than that of control rats; the lowering efficacy of dietary cycloalliin was a dose-dependent manner.

4. DISCUSSION

When growing male SD-rats were fed cholesterol-enriched atherogenic diets supplemented with or without cycloalliin for 2 weeks, no change in growth parameters such as body weight gain and food intake was found (Table 3-1-2), suggesting that cycloalliin, at least up to the 0.3 % dietary level, had no deleterious effect. We demonstrated in the present studies that feeding of cycloalliin, a cyclic sulfur imino acid found in onion, caused

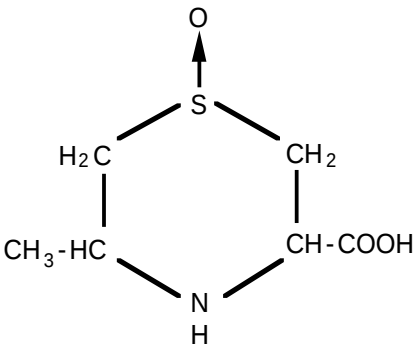
a marked reduction in the concentration of serum TAG in rats fed cholesterol-enriched diets. As hypertirglyceridemia is reported to be a risk factor of atherosclerosis (Brewer, 1999), cycloalliin in cooked onion is a beneficial ingredient for ameliorating lipid disorders caused by westernization of dietary habits. In the cycloalliin-fed rats, the reduced concentration of serum TAG was almost comparable at the levels of 0.1 % and 0.3 %, suggesting 0.1 % supplementation is enough to cause a marked reduction of serum TAG. In contrast, serum cholesterol and phospholipid levels remained unchanged following feeding of cycloalliin for 2 weeks. On the other hand, there was no significant difference in the concentration of hepatic lipids. Thus, the effects of dietary cycloalliin are mostly observed on serum TAG component, but not other lipid compositions. Liver is central organ regulating lipid and lipoprotein metabolism. Furthermore, in general, serum lipid levels are closely associated with an altered lipid metabolism in the liver (Angelin, 1993).

In this context, we measured hepatic lipid concentration and the relating enzyme activities involved in TAG biosynthesis. Dietary cycloalliin produced no remarkable effects on hepatic TAG content and other lipid parameters (Table 3-1-3). Hepatic TAG content is regulated by TAG biosynthesis, secretion of TAG into blood stream in the form of lipoproteins, or degradation of fatty acid in mitochondrial beta-oxidation (Gibbsons, 2001). In this experiment, we measured several marker enzyme activities for TAG synthesis such as microsomal PAP, which is a key enzyme for TAG synthesis (Fremont, 1996), and DGAT in the liver (Waggoner, 1995). In addition, the enzyme activities relating *de novo* fatty acid synthesis such as malic enzyme and G6PDH, which are the enzymes generating NADPH, was also determined. However, we were unable to find any

alterations in the activities of PAP, malic enzyme, and G6PDH in the livers of rats fed diets containing cycloalliin as compared to the control group. These observations suggest that the reduced concentration of serum TAG by dietary cycloalliin is not attributable to an alteration of hepatic TAG and fatty acid syntheses, but rather with other steps such as lipoprotein assembly or secretion in the liver.

It is known that hepatic VLDL secretion is positively related to various steps such as lipid synthesis, apoB100 production, and MTP activity (Jamil, 1998); the latest enzyme is a soluble protein that is localized in the lumen of the endoplasmic reticulum in the cells of liver and small intestines, and this protein is recently reported to be essential for transporting the newly synthesized lipid components to the site for forming apoB100-containig lipoproteins, since depletion of MTP activity has been reported to cause abetalipoproteinemia (Wetterau *et al.*, 1992). It was also observed that inhibition of VLDL assembly by MTP inhibitor causes fatty liver (Wang *et al*., 1999). We are therefore focusing whether feeding of cycloalliin alters hepatic MTP activity. Since dietary cycloalliin significantly decreased serum TAG concentration without altering TAG synthesis and its concentration in the liver, the remaining possible factor is whether MTP activity is responded negatively (Gordon *et al*., 1996). Consistent with our assumptions, we demonstrated for the first time that dietary cycloalliin inhibits MTP activity in the liver. In addition, the decreased concentration of serum TAG appeared to relate to the decrease in the MTP activity in the liver, so we further analyzed the relationship between concentration of TAG in the serum and MTP activity by using linear regression analysis. A very close negative correlation was evident between these two parameters ($r = 0.925$,

$p < 0.05$, Fig. 8). Therefore, the cycloalliin-dependent decrease in MTP activity leads to an impairment of apoB100-containing lipoprotein assembly. A slight increase in hepatic TAG concentration by cycloalliin feeding favors for the assumption, although more detailed mechanism by which cycloalliin inhibits MTP activity remains to be determined.



γ-Glutamyl-S-propylene-L-cysteine sulfoxide

↓ γ-Glutamyltranspeptidase → Glutamic acid

S-Propenyl-L-cysteine sulfoxide

↓ Heat

Cycloalliin

Fig.4. Chemical structure and the process of formation of cycloalliin

Table 3-1-1. Composition of experimental diet.

Ingredients	Control	Cycloalliin	
	0 %	0.1 %	0.3 %
Casein	20.0	20.0	20.0
DL-Methionine	0.3	0.3	0.3
Vitamin mixture	1.0	1.0	1.0
Mineral mixture	4.0	4.0	4.0
Cellulose	5.0	5.0	5.0
α-Corn starch	15.0	15.0	15.0
Choline bitartrate	0.2	0.2	0.2
Cholesterol	0.5	0.5	0.5
Sodium cholate	0.125	0.125	0.125
Fat (Beef tallow	5.0	5.0	5.0
Fat (Corn oil	5.0	5.0	5.0
Sucrose	43.875	43.775	43.575
Cycloalliin*	0.0	0.1	0.3

* Cycloalliin was supplemented in the form of cycloalliin hydrochloride monohydrate.

Table 3-1-2. Effect of dietary cycloalliin on the growth performance and the food intake.

Groups	Control	Cycloalliin	
	0 %	0.1 %	0.3 %
Body weight (g)			
Initial	130.1±4.1	133.1±2.9	130.2±2.8
Final	268.2±5.2	268.1±2.9	266.8±3.5
Liver weight			
total (g)	17.4±0.4	17.2±0.6	17.1±0.4
(g/100g B.W.)	6.5±0.1	6.4±0.2	6.4±0.1
Food intake			
(g/day)	20.0±0.5	19.7±0.3	20.6±0.4

Four-week-aged SD male rats fed the semipurified diets supplemented without (control) or with cycloalliin at the levels of either 0.1 % or 0.3 % for two weeks. Values are expressed as mean ± SE of six in each groups.

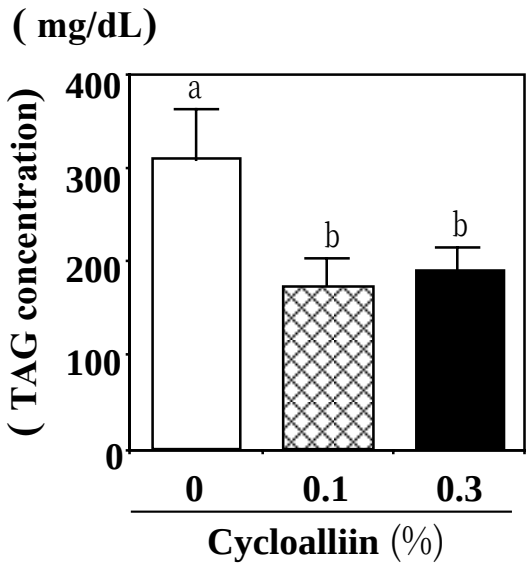


Fig.5. Effect of dietary cycloalliin on the serum TAG concentration.

Four-week-aged SD male rats fed the semipurified diets supplemented without (control) or with cycloalliin at the levels of either 0.1 % or 0.3 % for two weeks. Values are expressed as mean ± SE of six in each groups. Values with different letters are significantly different at $p < 0.05$.

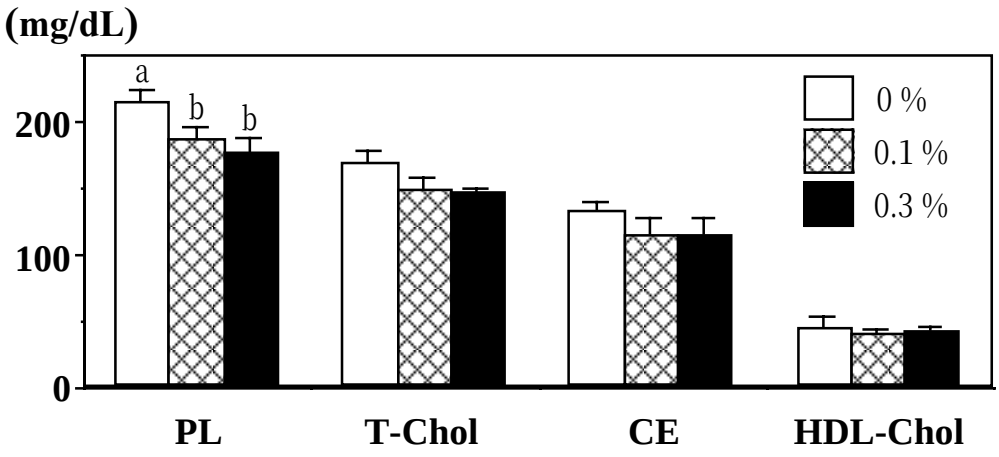


Fig.6. Effect of dietary cycloalliin on the serum lipid levels.
Four-week-aged SD male rats fed the semipurified diets supplemented without (control) or with cycloalliin at the levels of either 0.1 % or 0.3 % for two weeks. Values are expressed as mean ± SE of six in each groups. Values with different letters are significantly different at $p < 0.05$.
*Abbreviation: PL; phospholipid, T-Chol; total cholesterol, CE; cholesterol ester, HDL-chol; high density lipoprotein-cholesterol.

Table 3-1-3. Effect of dietary cycloalliin on the hepatic lipid levels.

Groups	Control	Cycloalliin	
	0 %	0.1 %	0.3 %
		(mg/g liver)	
Triacylglycerol	62.2±2.9	72.6±4.5	77.8±6.0
Phospholipid	21.9±1.9	25.9±0.8	24.2±1.9
Cholesterol	37.1±4.6	40.6±3.3	36.8±2.7

Four-week-aged SD male rats fed the semipurified diets supplemented without (control) or with cycloalliin at the levels of either 0.1 % or 0.3 % for two weeks. Values are expressed as mean ± SE of six in each groups.

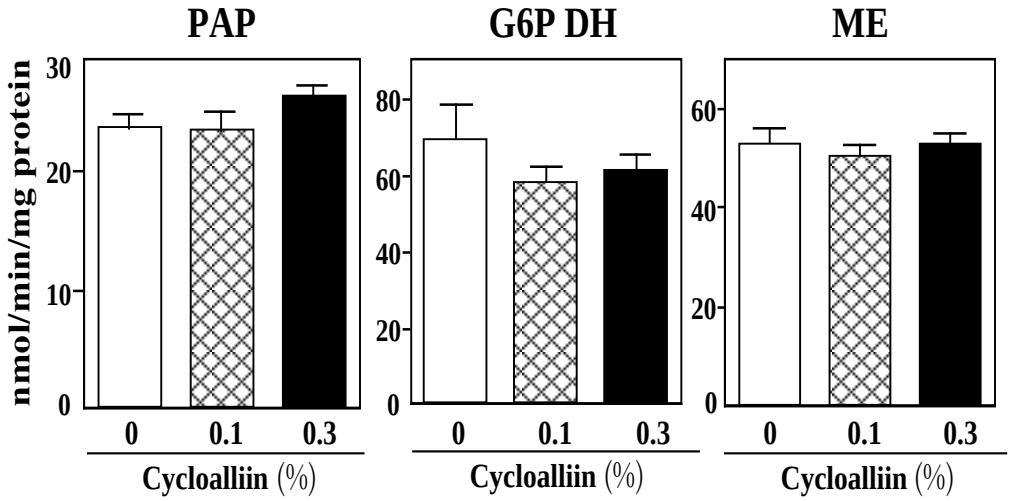


Fig.7. Effect of dietary cycloalliin on the activities of hepatic lipogenic enzymes. Four-week-aged SD male rats fed the semipurified diets supplemented without (control) or with cycloalliin at the levels of either 0.1 % or 0.3 % for two weeks. Values are expressed as mean ± SE of six in each groups. Values with different letters are significantly different at $p < 0.05$. *Abbreviation: PAP: phosphatidate phosphohydrolase, G6PDH: glucose-6-phosphate dehydrogenase, ME: malic enzyme.

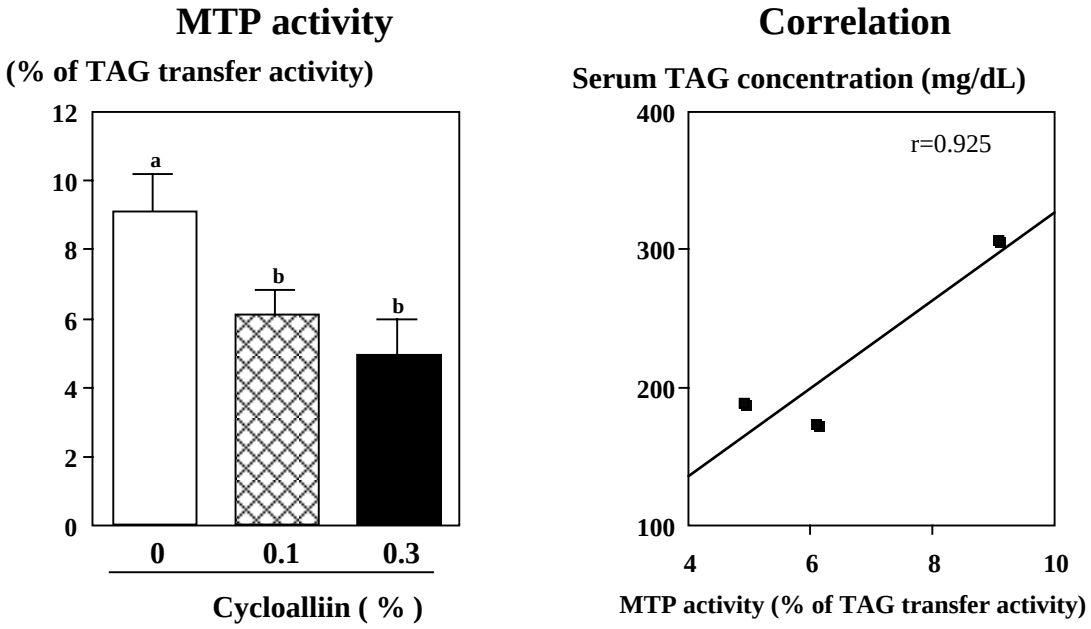


Fig. 8. Effect of dietary cycloalliin on the MTP activity.
Four-week-aged SD male rats fed the semipurified diets supplemented without (control) or with cycloalliin at the levels of either 0.1 % or 0.3 % for two weeks. Values are expressed as mean ±SE of six in each groups. Values with different letters are significantly different at $p < 0.05$. *MTP: microsomal TAG transfer protein.

Part 2. Effects of cycloalliin on lipid and apoB100-containing lipoprotein metabolism in HepG2 cells

1. INTRODUCTION

In Part 1 of this chapter, we have demonstrated that feeding of cycloalliin, main component of sulfur-containing compound of cooked onion, exhibits a hypotriglyceridemic action. This reduction was, in part, due to a reduced activity of hepatic MTP without altering the synthesis of TAG in the liver of rats. In this part, to understand a detailed mechanism responsible for the observed reduction of serum TAG level, an effect of cycloalliin on the lipoprotein metabolism in HepG2 cell was studied. HepG2 cells are derived from human hepatoblastoma and retains many original functions of normal human hepatocytes (Sandra, 1995). It is widely used to study the regulation of lipid and lipoprotein metabolism (Erickson & Fielding, 1995; Yanagita *et al*., 1995,1996,1999). In this part, we investigate whether an addition of cycloalliin to the culture medium of HepG2 cells alters the secretion of apoB100 and newly synthesized TAG from the liver.

2. MATERIALS AND METHODS

2.1 Experimental material and reagents

Cycloalliin (cycloalliin hydrochloride monohydrate) was supplied by Nippon Shinyaku (Kyoto, Japan). HepG2 cells were obtained from Riken Cell Bank (Tsukuba,

Japan). Dulbecco s Modified Eagle medium (DMEM) and trypsin were provided from Gibco (Grand Island, NY, USA). Fetal calf serum (FCS) was obtained from Sigma (St. Louis, MO, USA), and penicillin and streptomycin were products of Meiji Seika Kaisha, Ltd. (Tokyo, Japan). Lipoprotein-deficient serum (LPDS) was purchased from Sigma (St. Louis, MO, USA). [^{14}C]Acetate (specific activity: 2.07 MBq/μM) was purchased from Amersham, Inc. (Buckinghamshire, England). Scintillation cocktail (Sintisol Ex-H) was purchased from Dojin Chemical Company (Kumamoto, Japan).

2.2 HepG2 cell culture

HepG2 cells were preincubated in DMEM supplemented with 10 % FCS, penicillin (100 μg/mL), and streptomycin (100 μg/mL) at 37 °C in air containing 5 % (v/v) CO_2 to keep the pH 7.4 constant. The culture medium was renewed with fresh medium every third day. Semiconfluent cells on 24-well cell culture plate were washed twice with DMEM, and cultured for 24 h in the medium containing 10 % LPDS and 1 mM oleate with added cycloalliin. The cells cultured in the medium in the absence of cycloalliin were used as control. [^{14}C]Acetate (18.5 kBq) was added into the medium at the beginning of experiment. After incubation, cells and medium were harvested separately. After removal of culture medium, cells were washed twice with chilled phosphate buffered-saline (PBS), and then 1 mL of PBS was added into each well. The cells were collected, transferred into a rubber policeman, and frozen at -80 °C until analysis.

2.3 Preparation of experimental medium

Cycloalliin dissolved in dimethyl sulfoxide (DMSO) was diluted in 10 % LPDS-DMEM. Final concentration of cycloalliin in the medium was 1 μM or 100 μM. All

experimental mediums were sterilized by passing through a 0.45 μm Millipore R filter.

2.4 Determination of cellular protein concentration

Cells were disrupted with a sonicator (sonifier 250^{TM}, Branson Ultradonid Co., Connecticut, USA), and analyzed for cellular protein concentration by using the BCA protein assay kit purchased from PIERCE (Rockford, IL, USA) (Smith *et al*., 1985).

2.5 Cell viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed as a parameter of cell viability as reported by Sladowski *et al*. (1993), since it is known that HepG2 cells stained with MTT reagent indicate functionally normal. In brief, the cells were precultured in medium on a 96-well plate, and then 20 μL of MTT solution (5 mg/mL PBS) was added into each well. The cells were incubated for another 3 h, and then the reaction was terminated by addition of 150 μL of mixture (dimetyl sulfoxide:ethanol=1:1, v/v) following removal of medium. After shaking for 15 min, the absorbance was determined at 570 nm by the 96-well plate reader (CS-9300 PC, Simadzu Co., Ltd., Kyoto, Japan).

2.6 Accumulation of apoB100 in the medium by HepG2 cells

ApoB100 concentration in the medium was measured by a double antibody enzyme-linked immunosorbent assay (ELISA) as reported by Young *et al*. (1986). In brief, the medium collected was diluted 200-fold with PBS, and transferred to the 96-well plates which were coated with coating buffer containing 15 mM Na_2CO_3, 31 mM $NaHCO_3$, pH 9.6, and 17.3 mg/L of rabbit anti-human IgG; this was maintained at 37 °C for 2 h, and then each well was incubated with 0.1 mL of BSA containing 0.5 % sodium azide for

another 2 h. The wells were washed three times with PBS containing 0.5 mL/L of Tween 20. Sample solution was added to the wells, and incubated at 4 °C for 16 h. After washing, 0.1 mL of phenylenediamine substrate solution was added to the wells and maintained at 25 °C for 30 min. The reaction was terminated by adding 50 μL of 4.5 M H_2SO_4, and the absorbance was determined in the individual well at 492 nm by the 96-well plate reader.

2.7 TAG syntheses and their secretion into medium

The radioactivity of medium lipids were measured by a liquid scintillation counter (Wallic system 1410, Pharmacia, Sweden) as reported by Yanagita *et al*. (1995), following the extraction and purification of total lipids by the method of Bligh and Dyer (1959). Neutral lipid subclasses were separated by thin-layer chromatography using a precoated silica gel G plate developed with a solvent mixture of petroleum ether:diethylether:glacial acetic acid (80:20:1, v/v/v). Autoradiography of the plate was performed with an imaging plate and a BAS 1000 image analyzer (Fuji Photo Film Co., Ltd. Kanagawa, Japan).

2.8 Statistical analyses

Each value is presented as means ± SE. Data were analyzed by one-way ANOVA, all differences being inspected by Duncan s new multiple-range test (Duncan, 1955). A difference was considered significant at $p < 0.05$. Student s t-test (Fisher, 1970) was also used in some comparison.

3. RESULTS

3.1 HepG2 cell viability assay

HepG2 cells were cultured up to 24 h in the medium supplemented with or without cycloalliin. Final cycloalliin concentration was 1 μM or 100 μM. No significant change was noted in the concentration of intracellular protein following incubation with cycloalliin up to 100 μM (Fig.9). Cell viability also remained unchanged among the groups (Fig. 9). These results suggest that cycloalliin had no significant effect on cell viability.

3.2 Secretion of apoB100 by HepG2 cells

Figure 10 shows the apoB100 concentration in the medium after incubation with cycloalliin for 24 h. Cycloalliin treatment at the concentration of 1 μM as compared to the control caused a 24 % reduction in the accumulation of apoB100. The increasing amounts of cycloalliin from 1 μM to 100 μM had no further influence on apoB100 production, suggesting that treatment of 1 μM cycloalliin with HepG2 cells caused maximum rate of inhibition on apoB100 production (Fig. 10).

3.3 Incorporation of [^{14}C]acetate into medium TAG

Incorporation of [^{14}C]acetate into medium TAG was measured after 24 h incubation. Incorporation of [^{14}C]acetate into medium lipids was more marked into TAG than into both cholesterol ester and free cholesterol (data not shown). These results suggest that acetate added at the beginning of experiment was taken up by hepatocyte, and was secreted as lipids to the medium. Under this condition, cycloalliin treatment markedly decreased the secretion of [^{14}C]TAG (Fig. 12).

4. DISCUSSION

The present study focused on the effect of cycloalliin on TAG metabolism and apoB100 production in HepG2 cells. Incubation of the cells with cycloalliin at the concentration up to 100 μM had no effect on cell viability and cellular protein concentration compared with no added cycloalliin (Fig. 9). These results indicate that cycloalliin had no toxic effect on cell function under the present experimental conditions. Since apoB100 is a main protein component of lipoproteins, we measured apoB100 concentration in the medium. It was found that apoB100 production was decreased by the treatment of cycloalliin at the concentration of 1 μM, suggesting the decrease in the inhibition of VLDL assembly and synthesis. Furthermore, we examined the effect of cycloalliin on the secretion of newly synthesized TAG from the HepG2 cells by using radiolabeled precursor, [^{14}C]acetate. Acetate was employed for fatty acid synthesis and then TAG synthesis. In this condition, cycloalliin apparently reduced the secretion of labeled TAG level (Fig. 12). Intracellular apoB100 metabolism is regulated by the component of lipids, especially TAG or cholesteryl ester (Roger, 1996). The present observations suggest a high positive correlation between TAG and apoB100 concentrations in the medium (data not shown). Indeed, the reduction of apoB100 concentration in the medium was correlated well with the decrease in the concentration of TAG in the medium (Fig. 11). Considering together with these results from *in vivo* experiment with hypercholesterolemic rats and HepG2 cells *in vitro*, mechanism(s) responsible for hypotriglyceridemic action of dietary cycloalliin might be chain events in the liver as follows: a) decrease in transfer of TAG to the site for binding with apoB100 due to

decreased MTP activity; b) decreased synthesis and secretion of apoB100-containing lipoproteins; c) decreased the concentration of TAG in the serum.

In conclusion, the results presented in this chapter demonstrated for the first time that cycloalliin could reduce the concentration of serum TAG, and this hypotriglyceridemic action was, in part, due to the coordinated reduction of hepatic MTP activity and apoB100-containing lipoprotein assembly in the liver.

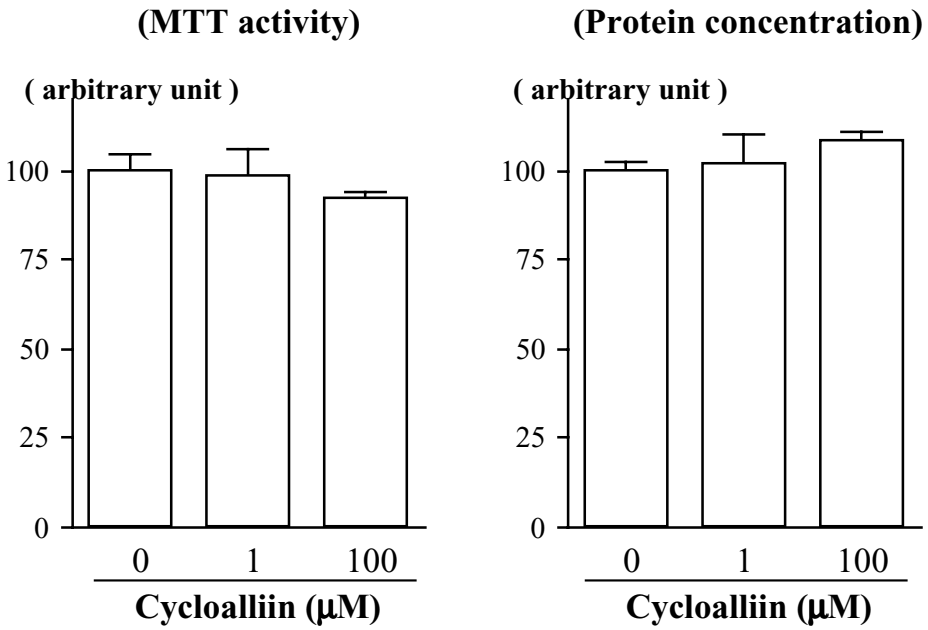


Fig.9. Effect of cycloalliin on the cytotoxicity and the cell proliferation.
Semiconfluent HepG2 cells were preincubated in the DMEM containing 10 % LPDS (basal medium) and 0.5 mM oleate for 24 h. Then, the cells were incubated in the basal medium containing 1 or 100 μM) of cycloalliin for another 24 h.
Data are expressed as a percentage of the control and represent means ± SE of 4 samples.

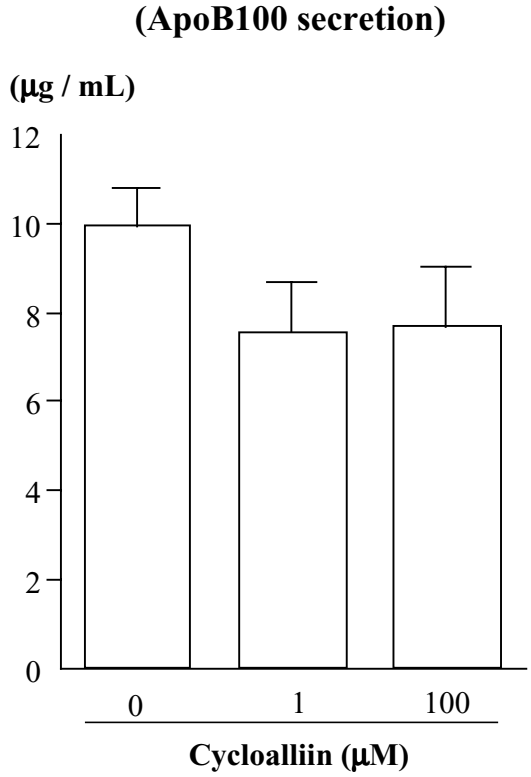


Fig.10. Effect of cycloalliin on apolipoprotein B100 secretion into the medium. Semiconfluent HepG2 cells were preincubated in the DMEM containing 10 % LPDS (basal medium) and 0.5 mM oleate for 24 h. Then, the cells were incubated in the basal medium containing 1 or 100 μM of cycloalliin for another 24 h. Data are expressed as a percentage of the control and represent means ± SE of 4 samples.

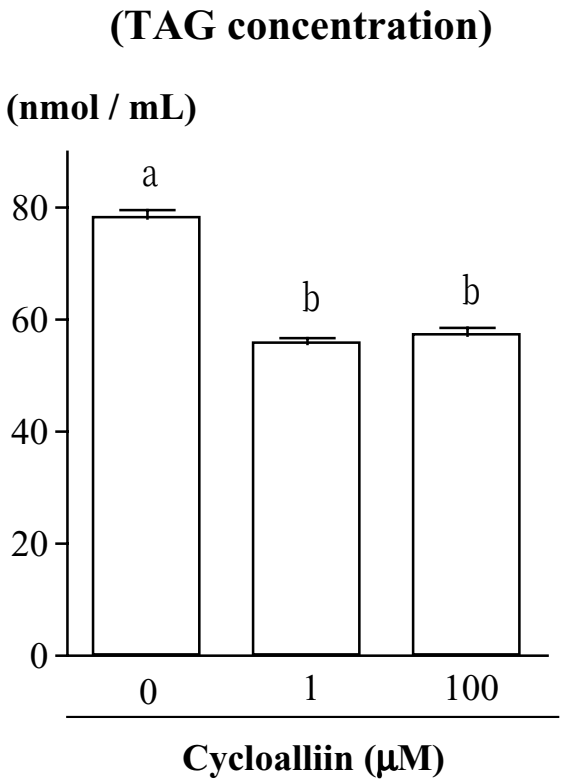


Fig.11. Effect of cycloalliin on the concentration of TAG in the medium.

Semiconfluent HepG2 cells were preincubated in the DMEM containing 10 % LPDS (basal medium) and 0.5 mM oleate for 24 h. Then, the cells were incubated in the basal medium containing 1 or 100 μM of cycloalliin for another 24 h. After extraction and separation by TLC, TAG was measured quantitatively by GLC. 15:0 TAG was used as an internal standard. Data are expressed as a percentage of the control and represent means ± SE of 4 samples. Values with different letters are significantly different at $p < 0.05$.

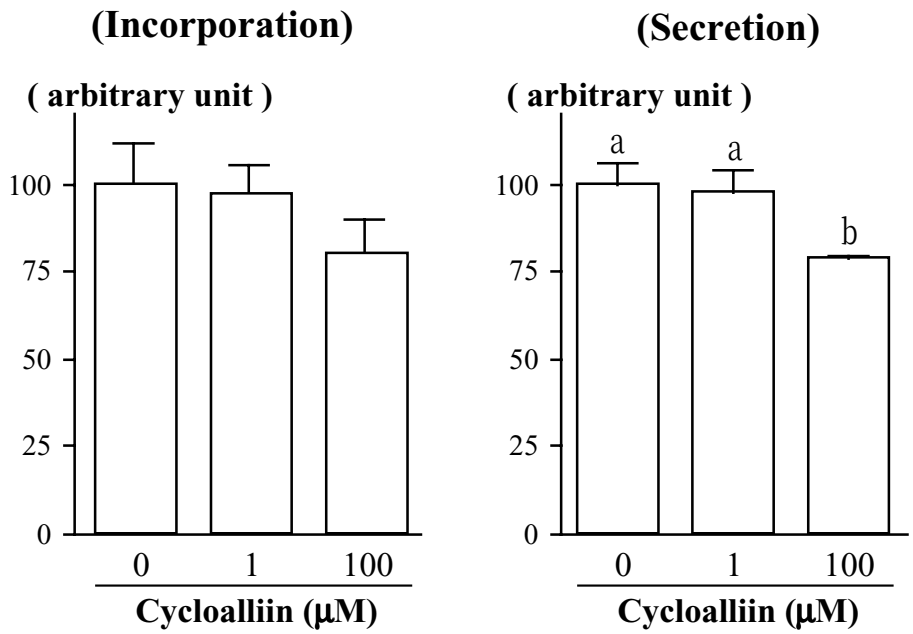


Fig12. Effect of cycloalliin on the incorporation of [^{14}C]acetate into TAG and secretion into the medium.

Semiconfluent HepG2 cells were preincubated in the DMEM containing 10 % LPDS (basal medium) and 0.5 mM oleate for 24 h. After preincubation, the cells were incubated in the medium containing 1 or 100 μM of cycloalliin and 18.5 kBq [^{14}C]acetate for 24 h. The value of the incorporation of [^{14}C]acetate into TAG in untreated cells ((657 ± 74) x 10^{-3} dpm/ mg cell protein) was arbitrarily defined as 100. The value of the secretion of [^{14}C]TAG into the medium in untreated cells ((47.1 ± 2.4) x 10^{-3} dpm/ mg cell protein) was arbitrarily defined as 100. Data represent means ± SE of 4 samples. Values not sharing the same letter are significantly different at $p < 0.05$.

CHAPTER IV

Effects of alkyl-cysteines and cysteine- and methionine-sulfoxides on lipid and apoB100-containing lipoprotein metabolism in HepG2 cells

Part 1. Effects of alkyl-cysteines on lipid and apoB100-containing lipoprotein metabolism in HepG2 cells

1. INTRODUCTION

In the previous chapter, we demonstrated that cycloalliin, a cyclic sulfur compound in onion, could reduce serum TAG concentration in rats fed the atherogenic diet. In addition, we also found that the addition of cycloalliin to the culture medium of HepG2 cells caused a reduction in the secretion of TAG and apoB100. These observations suggest that hypotriglyceridemic action of dietary onion extract is, in part, due to cycloalliin presented in this vegetable. On the other hand, it has been known that onion contains a variety of sulfur-containing compounds which are expected to have biological activities. However, the functions of these sulfur-containing amino acids were not extensively studied except for the relation to specific odor. Therefore we conducted to examine whether naturally occurring sulfur compounds found in onion have physiological activity. In this part, we examine whether alkyl-cysteines, S-methyl cysteine, S-ethyl cysteine, and S-propyl cysteine (Fig. 13) have effect on apoB100-containing lipoprotein metabolism in human liver cells.

2. MATERIALS AND METHODS

2.1 Experimental materials and reagents

HepG2 cells were obtained from Riken Cell Bank (Tsukuba, Japan). [^{14}C]Oleate (specific activity: 2.04 MBq/μM) and [^{14}C]acetate (specific activity: 2.07 MBq/μM) were purchased from Amersham, Inc. (Buckinghamshire, England). S-Methyl cysteine, S-ethyl cysteine, and S-propyl cysteine were purchased from Sigma (St. Louis, MO, USA). Scintillation cocktail (Sintisol Ex-H) was purchased from Dojin Chemical Company (Kumamoto, Japan). Other chemicals used in this part were the same as those described in the previous chapter.

2.2 HepG2 cell culture

HepG2 cells were treated with the same manner as those described in Chapter II and used for the following experiments.

2.2.1 Experiment I

The cells were cultured for 3 and 24 h in the medium supplemented with or without 10 μM S-propyl cysteine. [^{14}C]Acetate (18.5 kBq) or [^{14}C]oleate (18.5 kBq) was added into the medium at the beginning of experiment. After incubation, the cells and supernatant were harvested separately, stored in a rubber policeman, and frozen at -80 °C until analysis.

2.2.2 Experiment II

The cells were cultured for 24 h in the medium supplemented with or without one of the cysteine derivatives, S-methyl cysteine, S-ethyl cysteine, or S-propyl cysteine, at the concentration of 10 μM. [^{14}C]Acetate (18.5 kBq) was added into the medium at the beginning of the experiment, and chased as reported by Yanagita *et al*. (1995). After

incubation, the cells and the supernatants were harvested separately and frozen at -80 °C until analysis.

2.3 Preparation of experimental medium containing cysteine derivatives

Cysteine derivatives were dissolved in PBS, and diluted in DMEM containing 1 % BSA. All of these medium were sterilized by passing through a 0.45 μm Millipore R filter, and subjected to cell culture experiments.

2.4 Determination of cellular protein concentration

Cells were disrupted with a sonicator (sonifier 250^{TM}, Branson Ultradonid Co., Connecticut, USA) and subjected for the cellular protein concentration assay as described in Chapter II.

2.5 Cell viability

MTT assay was performed as described in Chapter II.

2.6 Determination of secretion of apoB100 into the medium from HepG2 cells

ApoB100 concentration was measured as described in Chapter II.

2.7 Lipid synthesis and their secretion into the medium

Incorporation of [^{14}C]acetate into cellular and medium lipids were measured as described in Chapter II.

2.8 Statistical Analyses

Each value is presented as means ± SE. Data were analyzed by one-way ANOVA, all differences being inspected by Duncan s new multiple-range test (Duncan, 1955). A difference was considered significant at $p < 0.05$. Student s t-test (Fisher, 1970) was also used in some comparison.

3. RESULTS

3.1 Effect of S-propyl cysteine on cell viability and protein concentration in HepG2 cells

As described in the previous chapter, cells stained with MTT reagent indicate the number of cells functioned normally. Therefore, this parameter can be used as an index of cell viability. S-Propyl cysteine treatment did not show any untoward effects on cell viability when treated with S-propyl cysteine at the concentrations in the range of 10 μM to 1000 μM (Fig. 14). Similarly, no significant difference was noted in the cellular protein concentration, after the 24 h-incubation of the cells with or without S-propyl cysteines (Fig. 14). These results therefore suggest that S-propyl cysteine at the concentrations used in this study is not cytotoxic to HepG2 cells.

3.2 Effect of S-propyl cysteine on the secretion of apoB100 in the medium

Figure 15 shows the concentration of secreted apoB100 in the medium. Accumulation of apoB100 in the medium was progressively increased with prolonged incubation; at the 24 h incubation, 1.7-fold higher apoB100 concentration in the medium was found in comparison with that for 3h. On the other hand, addition of S-propyl cysteine to the medium caused approximately 18-20 % reduction in apoB100 concentration in the medium at 3 and 24h-incubation. It clearly indicates that a S-propyl cysteine causes the reduction in the secretion of apoB100 by HepG2 cells.

3.3 Effect of S-propyl cysteine on the incorporation of [^{14}C]acetate into cellular and medium TAG and cholesterol

Incorporation of [^{14}C]acetate into cellular and medium lipids are shown in Fig. 16. Incorporation of [^{14}C]acetate into cellular TAG was similar in the presence and in the absence of S-propyl cysteine for 24 h. However, incorporation into medium was significantly lowered by 38 % following treatment of S-propyl cysteine (Fig. 16). Cells treated with S-propyl cysteine also exhibited a comparable incorporation rate of acetate into cellular cholesterol with those untreated cells (Fig. 16). However, accumulation of the labeled-cholesterol in the medium was again significantly lowered by the treatment of S-propyl cysteine. A similar response was observed for phospholipids (data not shown). These results suggest that S-propyl cysteine is a potent amino acid derivative to cause inhibitory effects on lipid secretion by the liver cells.

3.4 Effect of S-propyl cysteine on the esterification of [^{14}C]oleate to form TAG and cholesterol ester(CE)

Figure 17 shows incorporation of exogenous substrate [^{14}C]oleate into TAG and CE in the HepG2 cells. Incorporation of [^{14}C]oleate into TAG was far greater than that into CE in the liver (Fig. 17). It suggests that oleate supplied exogenously is taken up by the liver, and is predominantly incorporated into TAG rather than into CE, consistent with previous observations (Dixon *et al*., 1991). In this condition, addition of S-propyl cysteine to the medium caused 42 % and 34 % reductions in the incorporation of [^{14}C]oleate into cellular TAG and CE, respectively (Fig. 17). It might suggest that S-propyl cysteine inhibits the esterification process to form TAG and CE in hepatic cells, and hence lead to the reduction of apoB100-containing lipoprotein secretion by the liver. However, there may be possible other mechanism such as S-propyl cysteine added inhibits

the transport of exogenous oleate into the cells. It remains to clarify.

3.5 Effect of alkyl-cysteine derivatives on the accumulation of apoB100 and lipid syntheses

In the preceding experiment, we have observed that the treatment with S-propyl cysteine reduced accumulation of apoB100-containing lipoproteins in the medium of the cells. In the present study, we compared whether cysteine derivatives with various carbon chain length such as S-methyl cysteine, S-ethyl cysteine, and S-propyl cysteine, influence the secretion of apoB100-containing lipoproteins. In this experiment, HepG2 cells were incubated in the medium containing 10 μM of these cysteine derivatives.

Accumulation of apoB100 in the medium tended to decrease following treatment of increasing carbon chain length of cysteine derivatives, and a significant reduction was observed by the treatment of propyl-cysteine, the longest carbon chain length used (Fig.18). This suggests that carbon chain length of cysteine derivatives is an important factor for regulating apoB100 production in the liver.

Incorporation of the [^{14}C]acetate into TAG and cholesterol in the medium also tended to decrease by the treatment of these three cysteine derivatives (Fig. 19); the extent of reduction in incorporation of the labeled TAG into the medium was 20 %, 35 %, and 36 % for S-methyl cysteine, S-ethyl cysteine, and S-propyl cysteine, respectively. In addition, incorporation into free- and esterified- cholesterol was reduced in response to increasing carbon chain length of cysteine derivatives. Most remarkable reduction was again noted by the treatment of S-propyl cysteine. On the other hand, no differences were noted in incorporation of [^{14}C]acetate into cellular TAG, free- and esterified-cholesterol in

the cells treated with cysteine derivatives as compared to those without these derivatives.

4. DISCUSSION

The present study showed that S-propyl cysteine derived from onion could reduce apoB100 secretion by human liver cells, HepG2. High level of apoB100 is a risk factor for atherosclerosis, so the reduction of apoB100 secretion by cysteine derivatives may be beneficial for health. Factors that regulate the synthesis and secretion of apoB100-containing lipoproteins by liver cells are the availability of apoB100 and lipids and the activity of microsomal TAG transfer protein (MTP) (Craig *et al.*, 1988). ApoB100, a 556 kDa hydrophobic protein, is synthesized in the endoplasmic reticulum and shows LDL-receptor binding activity (Davis, 1999; Twisk *et al.*, 2000). After synthesis, it is stabilized through binding with lipids, and then is secreted as apoB100-containing lipoproteins. If insufficient lipid is available, apoB100 is unstable and undergoes degradation *via* the ubiquitin-proteasome pathway (Bonnardel and Davis, 1995; Fisher *et al.*, 1997). Therefore, the control of apoB100 expression in cells is considered to be post-transcriptional (Gordon *et al.*, 1996).

In HepG2 cells, S-propyl cysteine reduced the secretion of apoB100 compared to untreated cells (Fig. 15). ApoB100 is a major constituent of the lipoproteins produced in the liver. Therefore, the decreased secretion of apoB100 by HepG2 cells suggests that hepatic secretion of apoB100-containing lipoprotein (VLDL) would be reduced.

Exposure of HepG2 cells to S-propyl cysteine reduced the secretion of [^{14}C]TAG

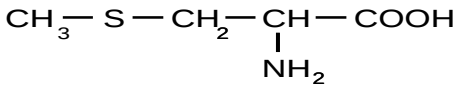
from [^{14}C]acetate significantly (Fig.16), in spite of the [^{14}C]acetate-incorporation into cellular lipids did not alter. These results suggest that S-propyl cysteine may inhibit the process of lipoprotein assembly and secretion. Liu and Yeh (2000) reported that S-propyl cysteine at high concentration (50-4000 μM) inhibited fatty acid synthesis and thereby TAG synthesis in cultured rat hepatocytes. The discrepancy on fatty acid synthesis by S-propyl cysteine between the present study and that by Liu and Yeh may be due to the difference of concentrations used and cells used (rat hepatocytes *versus* HepG2 cells). Cholesterol metabolism also affects on lipoprotein synthesis (Yanagita *et al*., 1999). In the present study, the incubation with S-propyl cysteine markedly reduced the secretion of [^{14}C]cholesterol from [^{14}C]acetate from the cells. However, the incorporation rate of [^{14}C]acetate into cellular cholesterol was not changed, although it was slightly lower with S-propyl cysteine treatment.

It is well recognized that MTP is an important factor involved in lipoprotein synthesis by the liver (Jamil *et al.*, 1997). MTP is a heterodimer consisting of a 97 kDa subunit with a lipid transfer activity and a 58 kDa subunit with a protein disulfide isomerase activity, and is essential for the transfer of lipid to nascent lipoproteins (Gregg and Wetterau, 1994). Complete absence of MTP causes abetalipoproteinemia (Harrity *et al.*, 1998; Wetterau *et al*., 1992), while MTP inhibitors are reported to inhibit the development of atherosclerosis in WHHL rabbits (Wetterau *et al.*, 1998). As described in Chapter III, cycloalliin, a cyclic imino compound found in onion, caused a marked reduction of the plasma TAG concentration. Therefore, it is necessary to perform investigations into the effect of sulfur amino acids on MTP activity and mRNA expression.

It is noteworthy that the inhibitory effect of the cysteine derivatives on apoB100 secretion from the cells increased in the order of S-propyl cysteine, S-ethyl cysteine, and S-methyl cysteine, indicating that the cysteine derivatives with a longer carbon chain had a stronger inhibitory effect (Fig. 18). An interaction of these compounds with the cell membrane and their entry into the cells may be responsible for this difference in their activity. As incubation with S-propyl cysteine resulted to reduce the cellular TAG and CE syntheses from [^{14}C]oleate, one might suppose that the sulfur compound inhibits the esterification of exogenous fatty acid to cellular lipids (Fig. 17). But, there was no any difference in cellular TAG synthesis from [^{14}C]acetate. Taken together, it is hypothesized that S-propyl cysteine may reduce the transport of exogenous fatty acids into the cells.

In conclusion, S-propyl cysteine from onion reduced apoB100 secretion by HepG2 cells derived from the human hepatoblastoma cells. Accordingly, the beneficial actions of foods including onion can be attributed to the presence of cysteine derivatives.

S- methyl -L-cysteine



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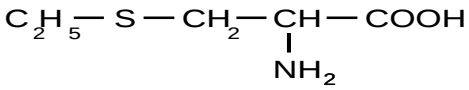

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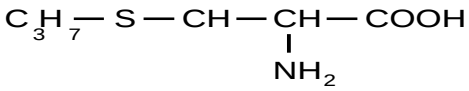

Fig.13. Chemical structures of cysteine derivatives

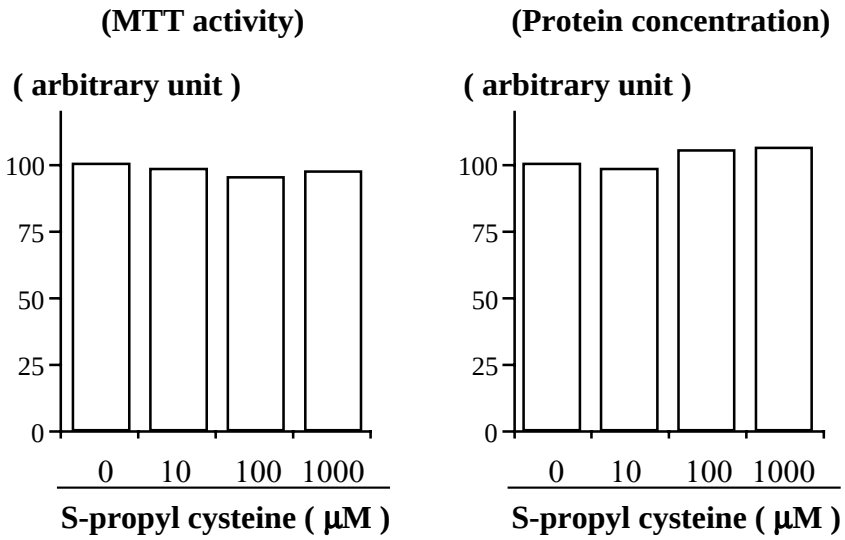

Fig.14. Effect of various concentrations of S-propyl cysteine on the cytotoxicity and cell proliferation.

Semiconfluent HepG2 cells were preincubated in the DMEM containing 1 % BSA (basal medium) and 0.5 mM oleate for 24 h. Then, the cells were incubated in the basal medium containing various concentrations (0 to 1000 μM) of S-propyl cysteine for another 24 h. Data are expressed as a percentage of the control and represent means ± SE of 4 samples.

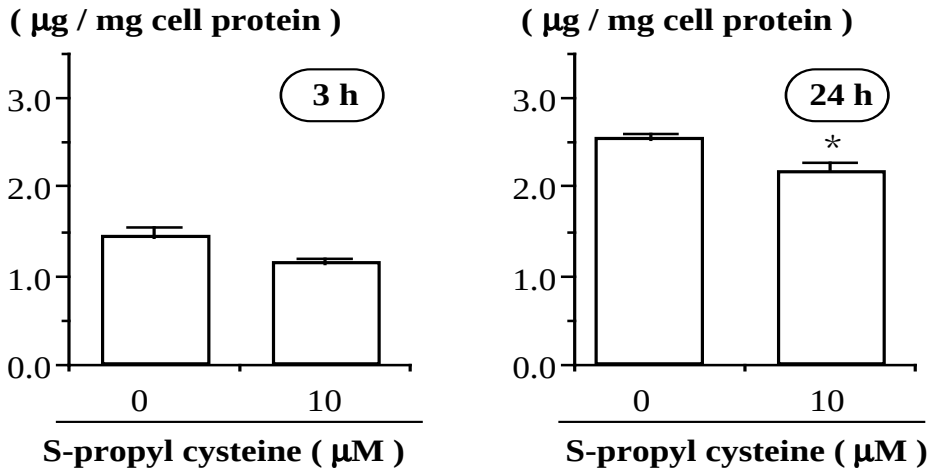

Fig.15. Effect of S-propyl cysteine on the apoB100 secretion from HepG2 cells. Semiconfluent HepG2 cells were preincubated in the DMEM containing 1 % BSA and 0.5 mM oleate for 24 h. Then the cells were incubated in the medium containing 10 μM of S-propyl cysteine for 3 and 24 h. After harvest, apoB100 content in the medium was measured by double antibody method, ELISA. Data represent means ± SE of 4 samples. * $p < 0.001$.

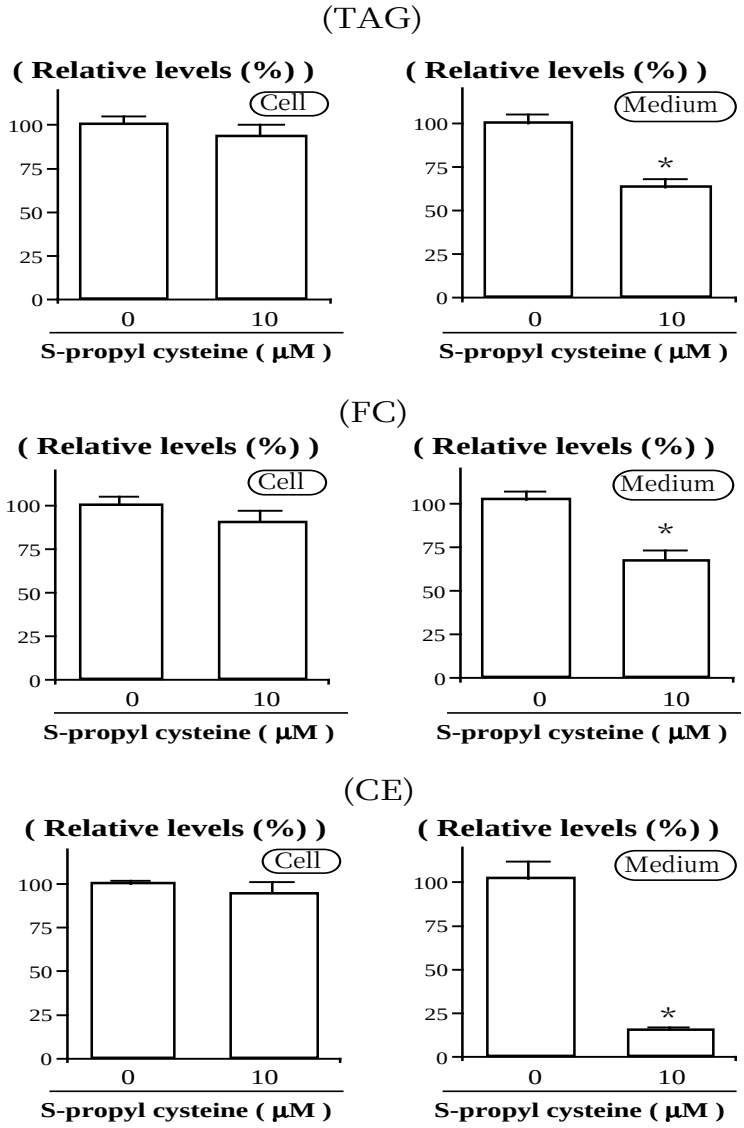

Fig.16. Effect of S-propyl cysteine on the incorporation of [^{14}C]acetate into TAG and cholesterols and secretion into the medium.

After preincubation with the DMEM containing 1 % BSA and 0.5 mM oleate for 24 h, the cells were incubated in the same medium containing 10 μM of S-propyl cysteine and 18.5 kBq [^{14}C]acetate for 24 h. See also Fig.15. The value of the incorporation of [^{14}C]acetate into TAG in untreated cells ((685.6 ± 27.5) x 10^{-3} dpm/mg cell protein) and the secretion of the labeled TAG into the medium

((16.1 ± 0.20) x 10^{-3} dpm/mg cell protein) were arbitrarily defined as 100, respectively. The value of the incorporation of [^{14}C]acetate into free cholesterol in untreated cells ((66.1 ± 2.41) x 10^{-3} dpm/mg cell protein) and the secretion of the labeled free cholesterol into the medium ((4.40 ± 0.24) x 10^{-3} dpm/mg cell protein) were arbitrarily defined as 100, respectively. The value of the incorporation of [^{14}C]acetate into cholesterol ester in untreated cells ((16.1 ± 0.25) x 10^{-3} dpm/mg cell protein) and the secretion of the labeled cholesterol ester into the medium ((0.44 ± 0.04) x 10^{-3} dpm/mg cell protein) were arbitrarily defined as 100, respectively. Data represent means ± SE of 4 samples. *Statistically significant difference from control at $p < 0.05$.

Abbreviation: TAG; Triacylglycerol, FC; Free cholesterol, CE; Cholesterol ester.

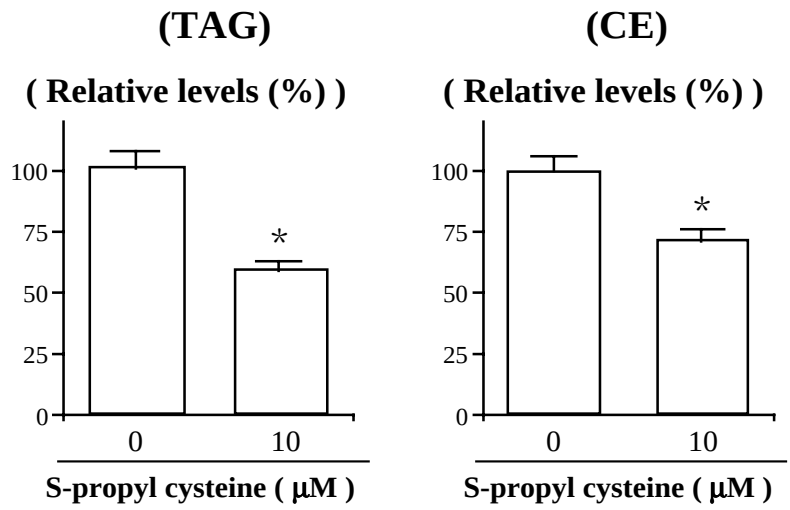

Fig.17. Effect of S-propyl cysteine on the incorporation of [^{14}C]oleate into TAG and cholesterol ester.

After preincubation with the DMEM containing 1 % BSA and 0.5 mM oleate for 24 h, the cells were incubated in the medium containing 10 μM of S-propyl cysteine and 18.5 kBq [^{14}C]oleate for 24 h. The value of the incorporation of [^{14}C]oleate into TAG in untreated cells ((422 ± 27.8) x 10^{-3} dpm/mg cell protein) was arbitrarily defined as 100. The value of the incorporation of [^{14}C]oleate into cholesterol in untreated cells ((5.71 ± 0.34) x 10^{-3} dpm/mg cell protein) was arbitrarily defined as 100. Data represent means ± SE of 4 samples. * $p < 0.05$. Abbreviation: TAG; Triacylglycerol, CE; Cholesterol ester.

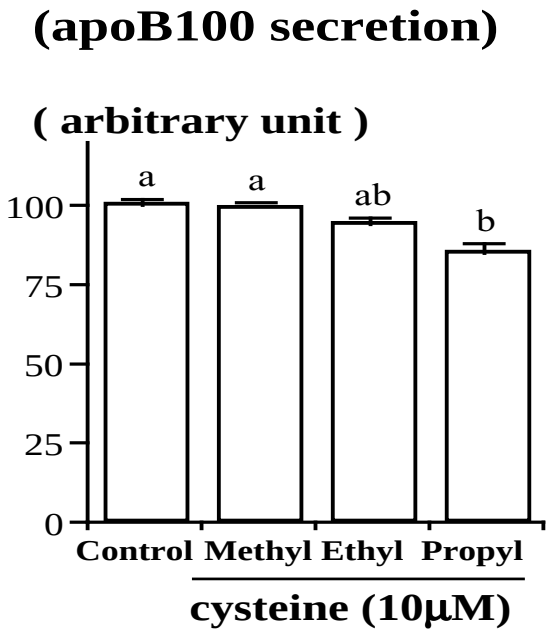

Fig.18. Effects of cysteine derivatives on the apoB100 secretion from HepG 2 cells.

After preincubation with the DMEM containing 1 % BSA and 0.5 mM oleate for 24 h, the cells were incubated in the medium containing 10 μM of cysteine derivatives, S-methyl-, S-ethyl-, and S-propyl cysteines for 24 h. After harvest, the secreted apolipoprotein B100 was measured by the method refered below Fig.14. The value of the secreted apoB100 into the untreated medium ((2.08 ± 0.11) μg/mg cell protein) was arbitrarily defined as 100. Data represent means ± SE of 4 samples. *Values not sharing the same letter are significantly different at $p < 0.05$.

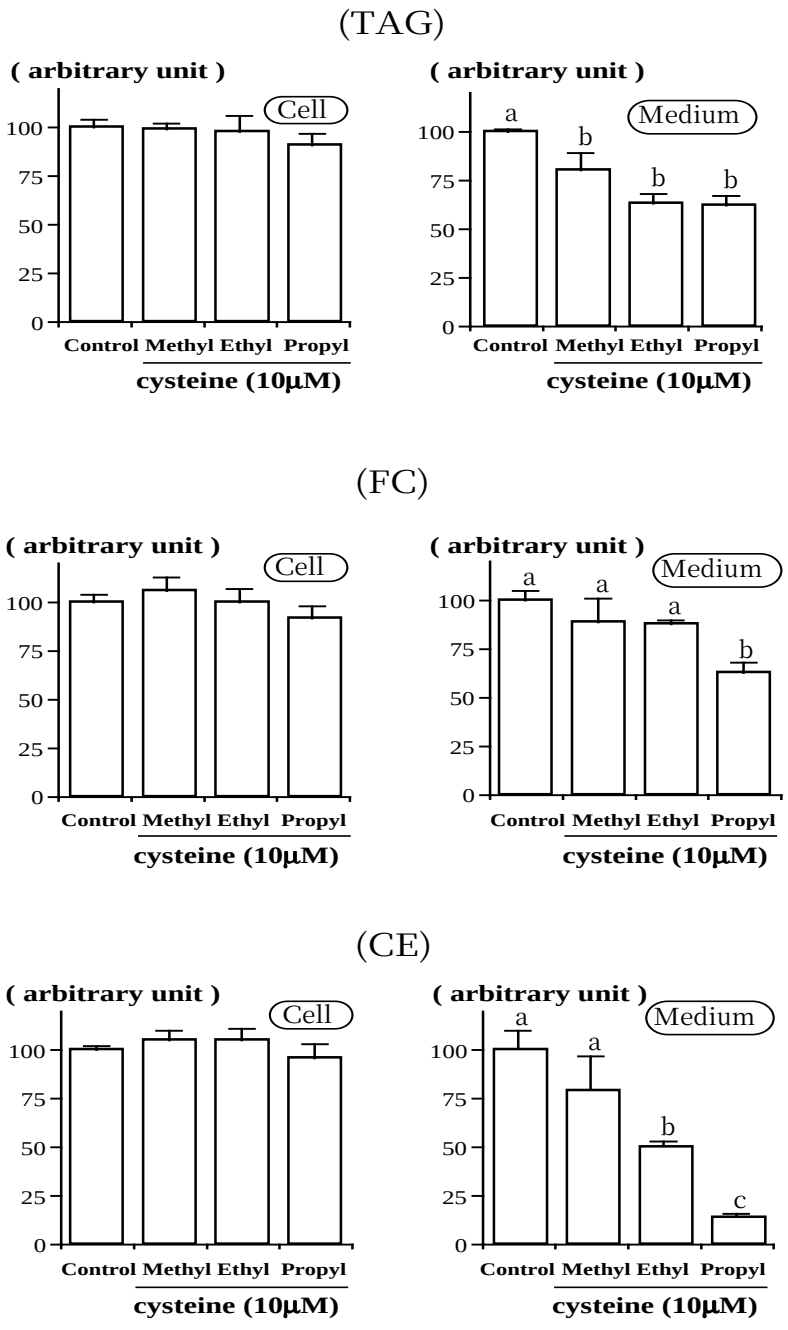

Fig.19. Effects of cysteine derivatives on the incorporation of [^{14}C]acetate into TAG and cholesterols and secretion into the medium.

After preincubation, the cells were incubated in the same medium (See legend in Fig.14) containing 10 μM of cysteine derivatives, S-methyl-, S-ethyl-, and S-propyl cysteines and 18.5 kBq [^{14}C]acetate for 24 h. The value of the

incorporation of [^{14}C]acetate into TAG in untreated cells and the secretion of the labeled TAG into the medium (as control) were arbitrarily defined as 100, respectively. The value of the incorporation of [^{14}C]acetate into free cholesterol in untreated cells and the secretion of the labeled free cholesterol into the medium (as control) were arbitrarily defined as 100, respectively. The value of the incorporation of [^{14}C]acetate into cholesterol ester in untreated cells and the secretion of the labeled cholesterol ester into the medium (as control) were arbitrarily defined as 100, respectively. Data represent means ± SE of 4 samples. *Values not sharing the same letter are significantly different at $p < 0.05$. Abbreviation: TAG; Triacylglycerol, FC; Free cholesterol, CE; Cholesterol ester.

Part 2. Effects of cysteine- and methionine- sulfoxides on lipid and apoB100-containing lipoprotein metabolism in HepG2 cells

1. INTRODUCTION

In previous parts, we reported that cycloalliin, a main sulfur-containing cyclic compound in cooked onion, markedly reduced serum TAG concentration in rats fed the atherogenic diet and, then the addition of cycloalliin to the culture medium reduced apoB100 secretion in HepG2 cells. In the previous part, S-propyl cysteine, S-ethyl cysteine, and S-methyl cysteine found in onion and garlic are also a potent inhibitor for apoB100 production in HepG2 cells. These results prompted us to examine whether other sulfur-containing compounds such as, propyl-cysteine sulfoxide, DL-methionine sulfoxide, S-carboxymethyl cysteine, and S-carboxyethyl cysteine (Fig. 20), found in onion of *Allium* species may also have biological activities such as alteration of apoB100-containing lipoproteins metabolism.

2. MATERIALS AND METHODS

2.1 Experimental materials and reagents

HepG2 cells were obtained from Riken Cell Bank (Tsukuba, Japan). DMEM and trypsin were provided from Gibco (Grand Island, NY, USA). The derivatives of

cysteine and methionine are supplied from Nippon Shinyaku, Ltd. (Kyoto, Japan). Other reagents and radioactive materials were purchased as descrebed in Chapter II.

2.2 HepG2 cell culture

The HepG2 cells were precultured in the DMEM containing 1 % BSA and 0.5 mM oleate for 24 h, and used for the experiments with the same methods as described in Chapter II.

2.2.1 Experiment I

The cells were cultured in medium supplemented with or without S-propyl-cysteine sulfoxide and DL-methionine sulfoxide, at the concentration of 10 μM for 24 h. As a lipid precursor, 37 kBq of [^{14}C]acetate was added into medium at the beginning of experiment. After incubation, the cells and the supernatants were harvested separately, and frozen at -80 °C until analysis.

2.2.2 Experiment II

The cells were cultured in the medium supplemented with or without S-carboxymethyl cysteine and S-carboxyethyl cysteine, at the different concentrations of 1-100 μM for 24 h. As a lipid precursor, 37 kBq of [^{14}C]acetate was added into the experimental medium at the beginning of experiment. After incubation, the cells and the supernatants were harvested separately, and frozen at -80 °C until analysis.

2.3 Preparation of experimental medium

Experimental mediums containing test amino compounds were sterilized after passing through a 0.45 μm Millipore R filter.

2.4 Determination of cellular protein

Cells were distrupted with a sonicator (sonifier 250^{TM}, Branson Ultradonid Co., Connecticut, USA), and the cellular protein concentration was determined as described in Chapter II.

2.5 Cell viability

MTT assay was performed as described in Chapter II.

2.6 Accumulation of apoB100 in the medium by HepG2 cells

ApoB100 concentration was measured by a double antibody enzyme-linked immunosorbent assay (ELISA) as described in Chapter II.

2.7 Lipid synthesis and secretion

The incorporation of [^{14}C]acetate into cellular lipids and their secretion were measured as described in Chapter II.

2.8 Statistical Analyses

Values are the means ± SE. Data were analyzed by one-way ANOVA, all differences being inspected by Duncan s new multiple-range test (Duncan, 1955). A difference was considered significant at $p < 0.05$.

3. RESULTS

3.1 Effect of S-propyl-cysteine sulfoxide and DL-methionine sulfoxide on apoB100 production (Experiment I)

Incubation with 10 μM of S-propyl-cysteine sulfoxide or DL-methionine

sulfoxide with HepG2 cells for 24 h did not cause any untoward effects on cell viability and intracellular protein concentration (Fig. 21). Figure 22 shows the effect of apoB100 production by HepG2 cells cultured in the presence or absence of these sulfoxides. Addition of S-propyl-cysteine sulfoxide and DL-methionine sulfoxide induced approximately 35 % reduction in the accumulation of apoB100 in the mediums.

3.2 Effect of S-propyl-cysteine sulfoxide and DL-methionine sulfoxide on the incorporation of [^{14}C] acetate into cellular and medium TAG and cholesterol

Incorporation of [^{14}C] acetate into TAG was comparable in the cells treated with S-propyl-cysteine sulfoxide and DL-methionine sulfoxide or without test reagent (control) for 24 h, while that into medium TAG was significantly lower in the cells treated with these two sulfoxides as compared to the control, the extent of reductions being 37 % and 55 % as compared to the control, respectively (Fig. 23). The ability to reduce incorporation of the label appeared to be higher in methionine sulfoxide than in propyl cysteine sulfoxide. Incorporation of [^{14}C]acetate into medium cholesterol (free and esterified) were significantly reduced by these sulfoxides (Fig. 24).

3.3 Effect of S-carboxymethyl cysteine and S-carboxyethyl cysteine on the incorporation of [^{14}C]acetate into TAG and cholesterol and their secretion (Experiment II)

Addition of S-carboxymethyl cysteine and S-carboxyethyl cysteine at the concentrations of 1 and 100 μM did not cause any deleterious effects on cell viability and protein concentration of HepG2 cells (Fig. 25). Incorporation of [^{14}C] acetate into cellular TAG following treatment with S-carboxymethyl cysteine and S-carboxyethyl cysteine as compared to those treated without cysteine derivatives for 24 h was comparable (Fig. 26).

On the other hand, incorporation of the label into medium TAG tended to be lower 25 % by S-carboxymethyl cysteine treatment, and was significantly lowered 48 % in 1 μM S-carboxyethyl cysteine (Fig. 26), suggesting an importance of chain-length of carbxyalkyl-chain bound to cysteine on the synthesis of TAG. When the cells were incubated with 100 μM S-carboxymethyl cysteine, the incorporation of the label was less than 50 % of the control value. With regard to cholesterol metabolism, effects of added cysteine derivatives on the incorporation of [^{14}C]acetate into cellular free- and cholesterol-cholesterol were equivocal, whereas that into medium cholesterol counterpart were apparent at the higher concentration of cysteine derivatives (Fig. 27). Thus, incorporation of label into free as well as cholesterol ester in the medium were significantly lowered by the treatment of both S-carboxyalkyl cysteines.

4. DISCUSSION

The present study demonstrated that derivatives of cysteine and methionine, S-propyl-cysteine sulfoxide, DL-methionine sulfoxide, S-carboxymethyl cysteine and S-carboxyethyl cysteine found in onion and garlic, lower the secretion of apoB100 and lipids in human liver cell model, HepG2.

It is known that liver is the pivotal organ concerned with lipid metabolism and apoB100-containing lipoproteins assembly and secretion (Angelin and Gibbsons, 1993). Overproduction of apoB100-containing lipoprotein in the liver results in hyperlipidemia (Loscalzo and Sniderman, 1993). Furthermore, higher concentrations of serum

cholesterol, TAG, and apoB100 are major risk factors on CHD and atherosclerosis (Brewer, 1999).

HepG2 cells treated with S-propyl-cysteine sulfoxide and DL-methionine sulfoxide reduced the accumulation of apoB100 in the cultured medium (Fig.22) compared with the untreated cells. ApoB100 is synthesized in the endoplasmic reticulum and assembled as components of apoB100-containing lipoproteins (Craig *et al*., 1988; Hebbachi *et al*., 1999). Following synthesis, apoB100 is stabilized by binding to lipids, which is then secreted as apoB100-containing lipoproteins. If insufficient lipid is available, apoB100 is unstable and undergoes degradation *via* the ubiquitin-proteasome pathway (Fisher *et al*., 1997). Therefore, it is believed that the control of apoB100 expression in cells is mainly post-transcriptional (Goldberg and Ginsberg, 1997; Gordon *et al*., 1996). It is known that VLDL assembly and secretion is dependent on the levels of TAG and cholesterol ester, the rate of apoB100 catabolism after translation, and MTP activity (Yanagita *et al*., 1999). To evaluate the mechanism by which apoB100 levels are reduced by the test compounds, we studied the cellular lipid synthesis and secretion by using [^{14}C]acetate as the lipid precursor.

S-Propyl-cysteine sulfoxide and DL-methionine sulfoxide significantly reduced TAG secretion to the medium as shown in Fig. 23 and showed no effect on the incorporation rate of [^{14}C]acetate into cellular TAG. The secretion of free cholesterol and cholesterol ester were reduced significantly by the treatment with two sulfoxides as shown in Fig. 24. S-Carboxymethyl cysteine and S-carboxyethyl cysteine also reduced labeled TAG, free cholesterol, and cholesterol ester secretion into the cultured medium (Figs.26

and 27). However, cells treated with S-carboxyalkyl cysteines also had no effect on the incorporation of [^{14}C]acetate into cellular TAG, free cholesterol and cholesterol ester. These results raised the possibility that these compounds interfere with lipoprotein assembly and secretion in the endoplasmic reticulum and Golgi apparatus.

MTP is necessary for the transfer of TAG, cholesterol and phospholipid, and binding of apoB100, thus regulates the synthesis and secretion of nascent lipoproteins (Jamil *et al*., 1998). MTP is a soluble protein that is localized to the lumen of the endoplasmic reticulum in cells from the liver and small intestine. It exists as a 97 kDa molecule with a lipid transfer activity or as a heterodimer of 58 kDa having an isomerase activity (Erickson and Fielding, 1995). Depletion of MTP activity causes abetalipoproteinemia (Wetterau *et al*., 1997; Wetterau *et al*., 1992). In the previous study, we showed that cycloalliin inhibited MTP activity in the liver, decreased serum TAG level and apoB100 secretion (Wetterau *et al*., 1998). We also showed that S-propyl cysteine and S-ethyl cysteine found in onion reduced apoB100 secretion in HepG2 cells in the previous part.

The exact mechanism by which S-propyl-cysteine sulfoxide and DL-methionine sulfoxide reduced apoB100 and lipid secretion remained to be determined. It is possible that these compounds may inhibit MTP activity in the liver and thus interfere with assembly and secretion of apoB100-containing lipoproteins in the liver. It is also necessary to investigate whether these sulfur-containing amino compounds alter MTP activity and MTP mRNA expression.

In conclusion, the present study demonstrated that sulfur-amino acid derivatives

in *Allium* species inhibited the secretion of apoB100 and lipids from the liver, which suggests that they may be beneficial in atherosclerosis.

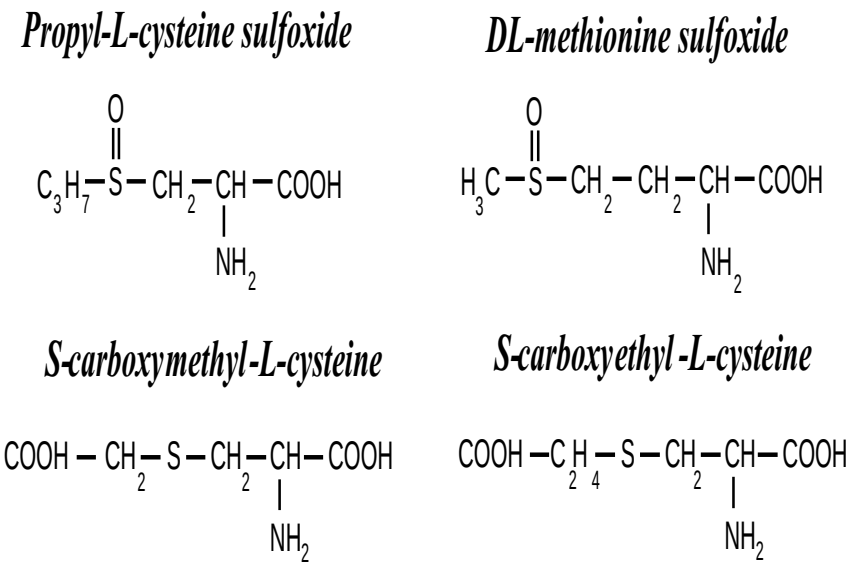

Fig.20. Chemical structures of the derivatives of cysteine and methionine.

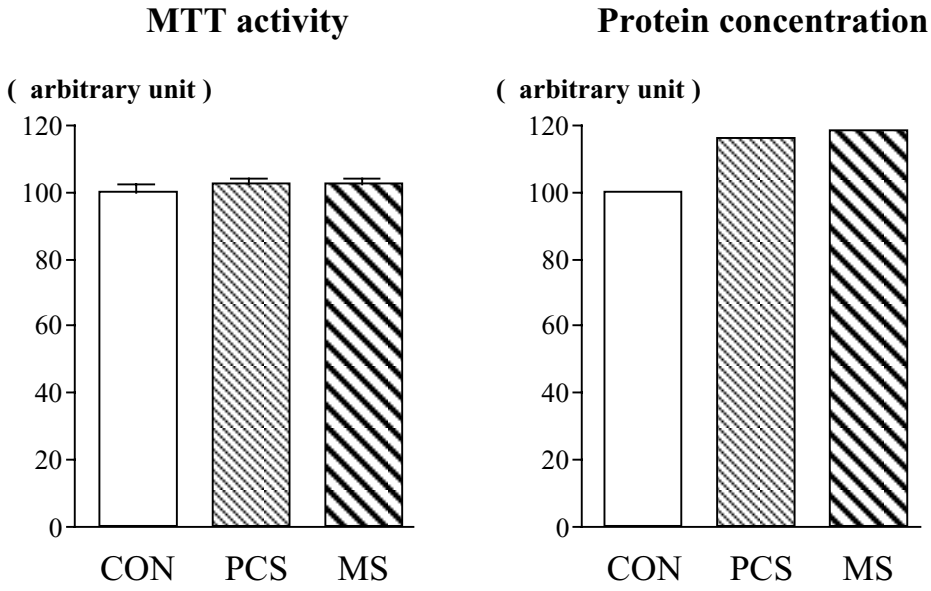

Fig.21. Effects of propyl-cysteine sulfoxide and DL-methionine sulfoxide on the cytotoxicity and cell proliferation.

Semiconfluent HepG2 cells were preincubated in the DMEM containing 1 % BSA (basal medium) and 0.5 mM oleic acid for 24 h. After preincubation, the cells were incubated in the basal medium containing 10 μM of propyl-cysteine sulfoxide or DL-methionine sulfoxide for another 24 h. The effect of sulfoxides on the cytotoxicity and cell proliferation were determined by MTT activity and cellular protein concentration. Data are expressed as a percentage of the control and represent means ± SE of 4 samples. *Abbreviation: CON; control, PCS; propyl-cysteine sulfoxide, MS; DL-methionine sulfoxide.

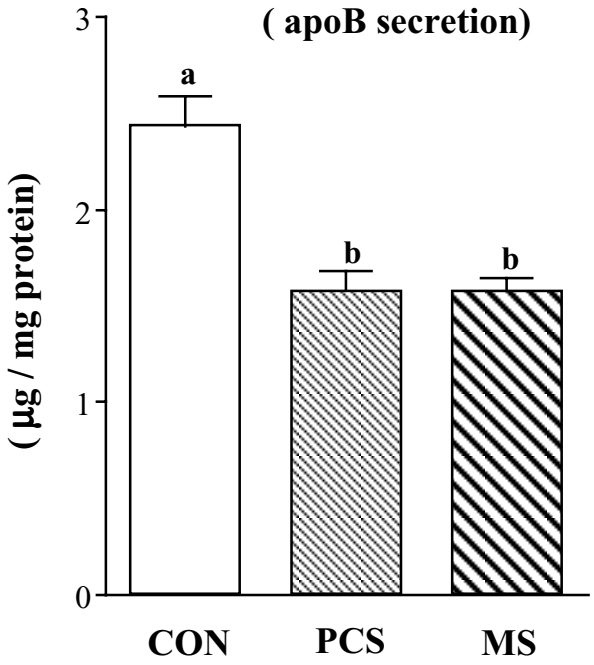

Fig.22. Effects of propyl-cysteine sulfoxide and DL-methionine sulfoxide on the apoB100 secretion from HepG2 cells.
Semiconfluent HepG2 cells were preincubated in the DMEM containing 1 % BSA and 0.5 mM oleic acid for 24 h. And then the cells were incubated in the medium containing 10 μM of propyl-cysteine sulfoxide or DL-methionine sulfoxide for 24 h. After harvest, the secreted apoB100 was measured by double antibody method, ELISA. Data represent means ± SE of 4 samples. * Values with different letters are significantly different at $p < 0.001$. Abbreviation: CON; control, PCS; propyl- cysteine sulfoxide, MS; DL-methionine sulfoxide.

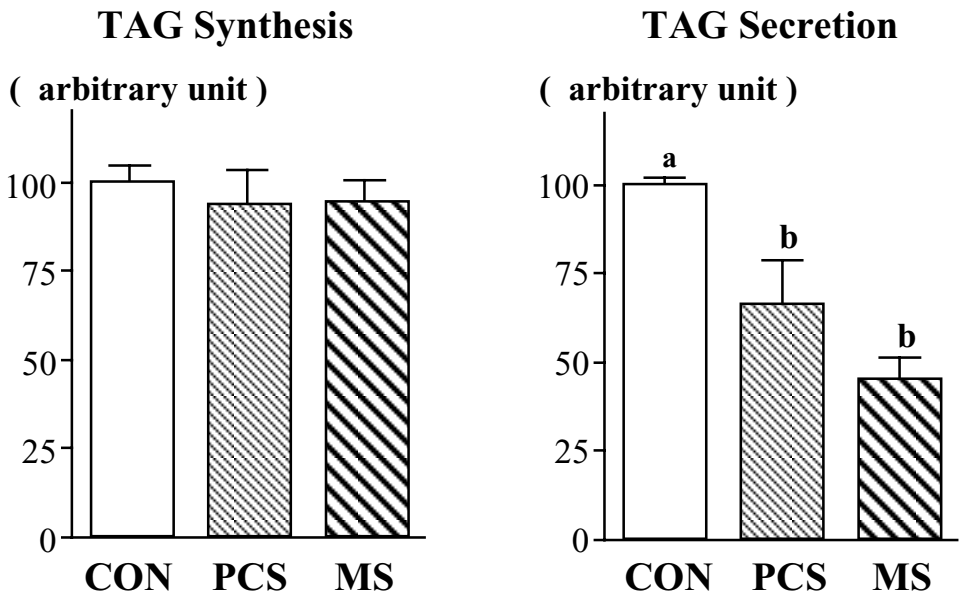

Fig.23. Effects of propyl-cysteine sulfoxide and DL-methionine sulfoxide on the incorporation of [^{14}C]acetate into TAG secretion into the medium.
After preincubation, the cells were incubated in the medium containing 10 μM of propyl-cysteine sulfoxide or DL-methionine sulfoxide and 37 kBq [^{14}C]acetate for 24 h. The value of the incorporation of [^{14}C]acetate into TAG in untreated cells and the secretion of the labeled TAG into the medium (as control), 686 ± 27 and 16.1 ± 0.20 (x 10^{-3} dpm/mg cell protein) were arbitrarily defined as 100, respectively. Values with different letters are significantly different at $p < 0.05$
*Abbreviation: CON; control, PCS; propyl-cysteine sulfoxide, MS; DL-methionine sulfoxide.

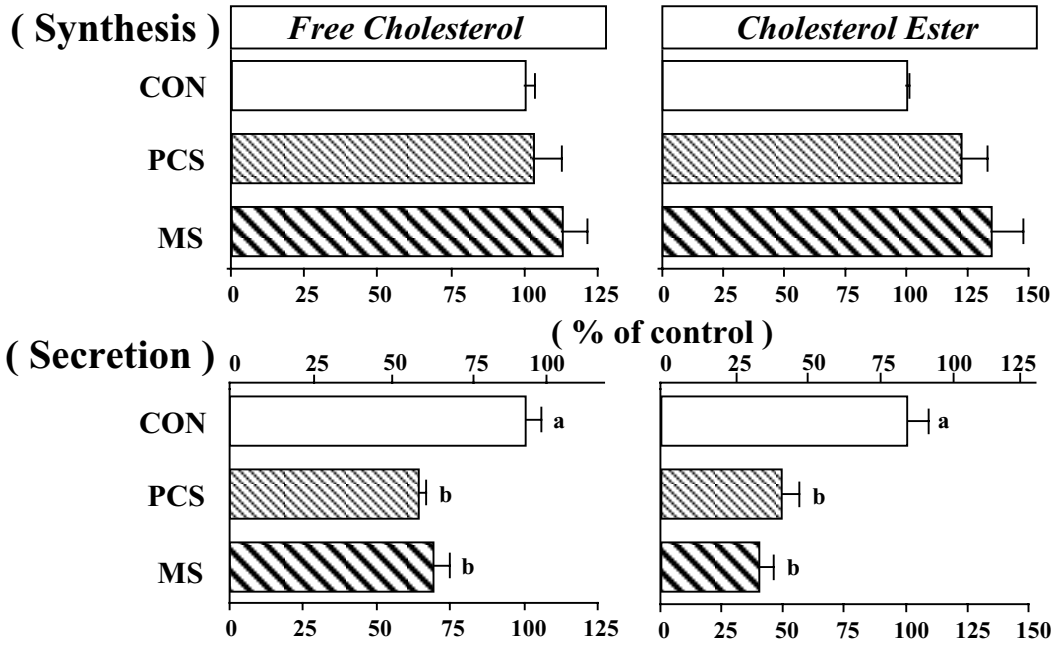

Fig.24. Effects of propyl-cysteine sulfoxide and DL-methionine sulfoxide on the incorporation of [^{14}C]acetate cholesterols and secretion into the medium.
The value of the incorporation of [^{14}C]acetate into free cholesterol in untreated cells and the secretion of the labeled free cholesterol into the medium (as control) , 66.1 ± 2.41 and 4.40 ± 0.24 (x 10^{-3} dpm/mg cell protein) were arbitrarily defined as 100, respectively. The value of the incorporation of [^{14}C]acetate into cholesterol ester in untreated cells and the secretion of the labeled cholesterol ester into the medium (as control), 16.1 ± 0.25 and 0.44 ± 0.04 (x 10^{-3} dpm/mg cell protein) were arbitrarily defined as 100, respectively. * Values with different letters are significantly different at $p < 0.05$. Abbreviation: CON; control, PCS; propyl-cysteine sulfoxide, MS; DL-methionine sulfoxide.

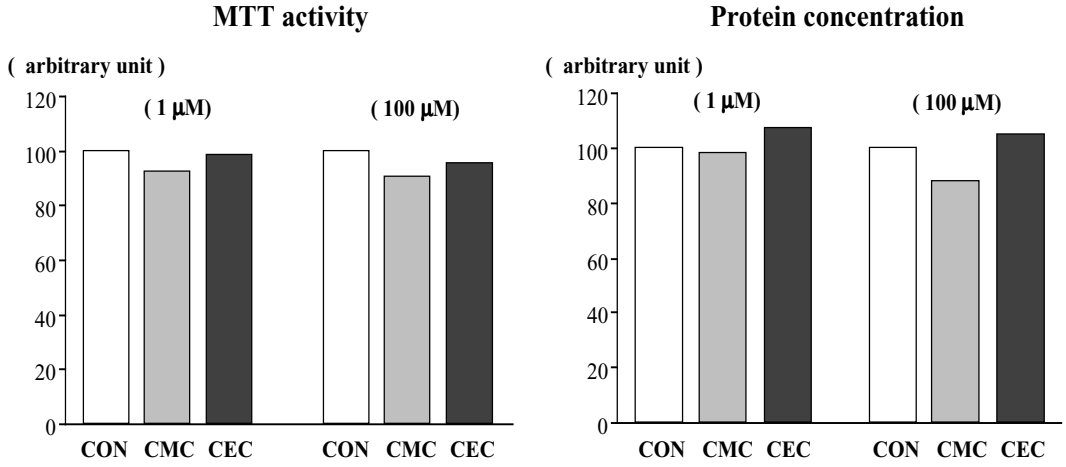

Fig.25. Effects of S-carboxyalkyl cysteines on the cytotoxicity and cell proliferation. Semiconfluent HepG2 cells were preincubated in the DMEM containing 1 % BSA (basal medium) and 0.5 mM oleic acid for 24 h. After preincubation, the cells were incubated in the basal medium containing 1, 100 μM of S-carboxyalkyl cysteines for another 24 h. The effect of S-carboxyalkyl cysteines on the cytotoxicity and cell proliferation were determined by MTT activity and cellular protein concentration. Data are expressed as a percentage of the control and represent means ± SE of 4 samples. *Abbreviation: CON; control, CMC; carboxymethyl cysteine, CEC; carboxyethyl cysteine.

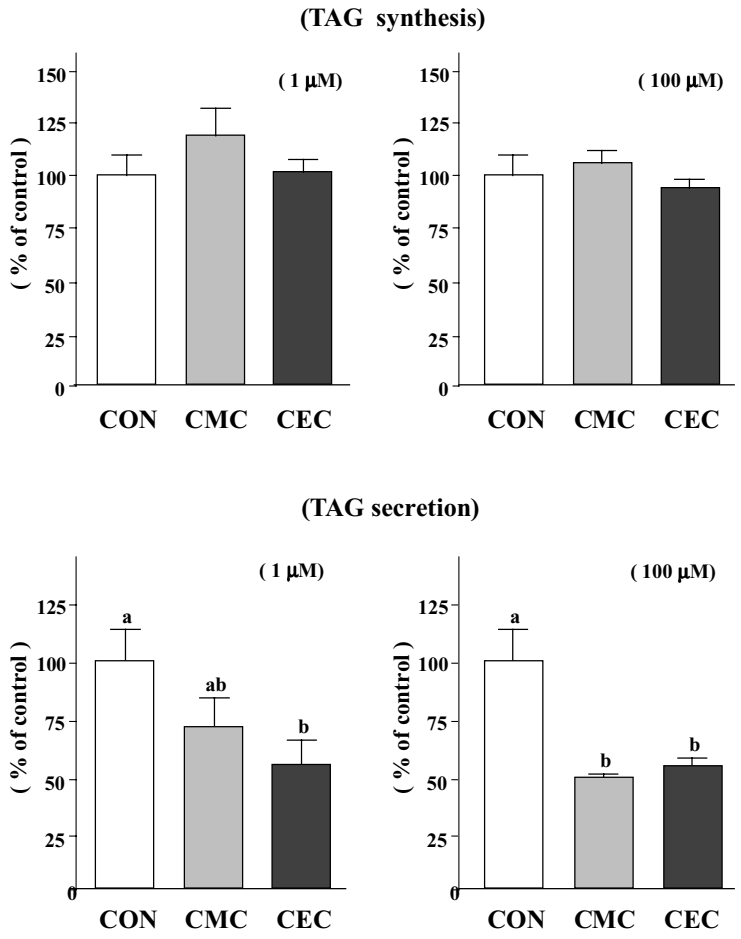

Fig.26. Effects of S-carboxyalkyl cysteines on the incorporation of [^{14}C]acetate into TAG and secretions into the medium.

After preincubation, the cells were incubated in the medium containing 1, 100 μM of S-carboxyalkyl cysteines and 37 kBq [^{14}C]acetate for 24 h. The value of the incorporation of [^{14}C]acetate into TAG in untreated cells (as control), 628 ± 63 (x 10^{-3} dpm/mg cell protein) was arbitrarily defined as 100, respectively. The value of the secretion of the labeled TAG into the medium (as control) , 13.6 ± 1.8 (x 10^{-3} dpm/mg cell protein) was arbitrarily defined as 100, respectively. *Values with different letters are significantly different at $p < 0.05$. Abbreviation: CON; control, CMC; carboxymethyl cysteine, CEC; carboxyethyl cysteine.

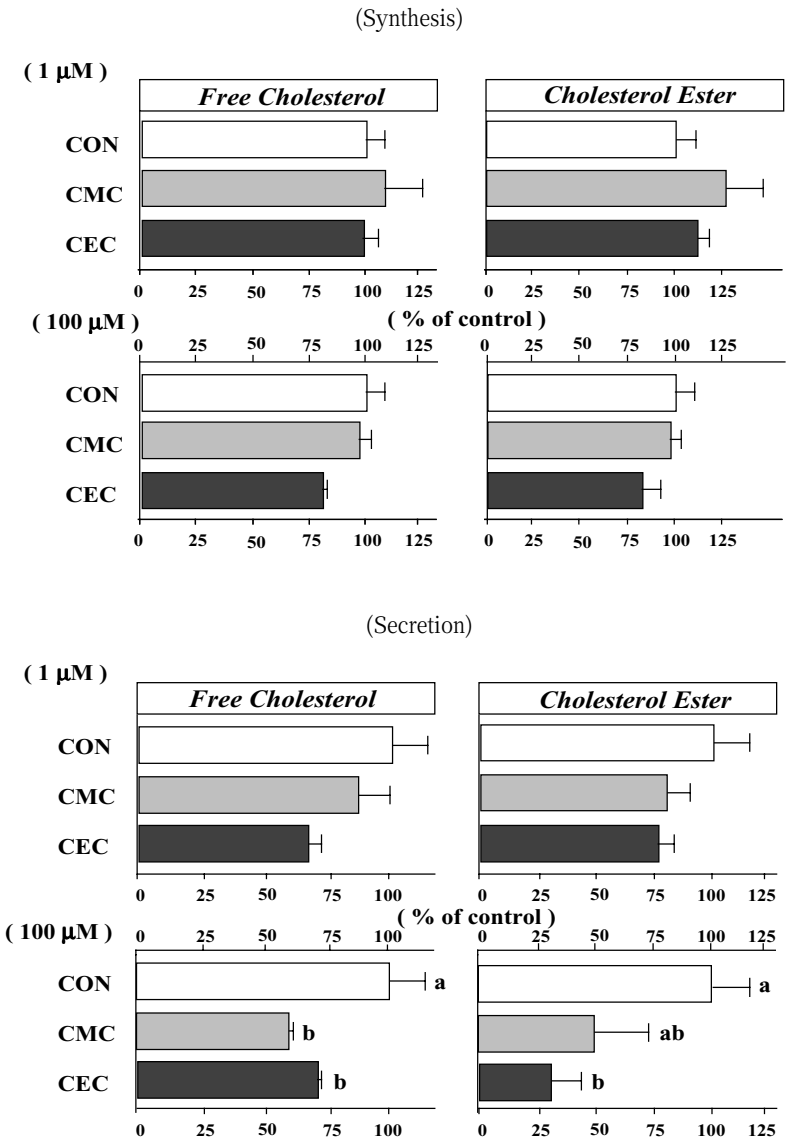

Fig.27. Effects of S-carboxyalkyl cysteines on the incorporation of [^{14}C]acetate into cholesterols and secretion into the medium.

The value of the incorporation of [^{14}C]acetate into free cholesterol in untreated cells and the secretion of the labeled free cholesterol into the medium (as control) , 75 ±7 and 8.5 ± 1.2 (x 10^{-3} dpm/ mg cell protein) were arbitrarily defined as 100, respectively. The value of the incorporation of [^{14}C]acetate into cholesterol ester in untreated cells and the secretion of the labeled cholesterol ester into the medium (as control), 19.2 ± 1.9 and 0.33 ± 0.05 (x 10^{-3} dpm/ mg cell protein) were arbitrarily defined as 100, respectively. * Values with different

letters are significantly different at $p < 0.05$. Abbreviation: CON; control, CMC; carboxymethyl cysteine, CEC; carboxyethyl cysteine.

CHAPTER V

SUMMARY & CONCLUSIONS

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Along with changes in dietary habits and lifestyle, various lifestyle-related diseases have been increasing, such as hyperlipidemia, obesity, diabetes, and hypertension, which all involve abnormalities of lipid metabolism. Vegetables and fruits are important nutrients for humanbeings and some of them contain various ingredients that exhibit physiological effects. Liliaceous plants (*Allium species*) such as onions and garlic have been long used as traditional medicines and are thought to have various effects, including prevention of heart diseases. It is reported that they contain abundant amounts of sulfur-containing compounds. In the preliminary study, we found that dietary onion reduced serum TAG level and adipose tissue weight.

To extend their therapeutic application and also to find the component responsible for the nutraceutical actions, we investigated the effects of several sulfur-containing compounds on the lipid and lipoprotein metabolism in experimental animals and human hepatoma cell line, HepG2.

In Chapter II, we investigated that dietary onion extract on tissue weight and lipid metabolism in rats. We found that dietary onion extract exerts for a hypotriglyceridemic action which was associated with a decrease in adipose tissue weights. in the rat fed atherogenic diet

In Chapter III, we evaluated that dietary cycloalliin, a cyclic sulfur-containing imino acid, on lipid and the related enzyme activities in rats and in HepG2 cells.

Cycloalliin reduced serum TAG level and the activity of microsomal TAG transfer protein, a factor for regulating the synthesis and secretion of apoB100-containing lipoproteins, VLDL. Furthermore, we found that cycloalliin reduced apoB100 and TAG secretion in HepG2 cells. Taken together, cycloalliin appears to inhibit VLDL assembly in the liver.

In Chapter IV, we evaluated that the effect of several derivatives of cysteine and methionine found in onion on lipid and apoB100-containing lipoprotein metabolism in HepG2 cells. The addition of S-methyl-, S-ethyl-, and S-propyl-cysteines, propyl-cysteine sulfoxide and DL-methionine sulfoxide resulted to reduce markedly TAG and apoB100 secretion to the medium. S-Carboxymethyl- and S-carboxyethyl cysteines also reduced TAG, free cholesterol, and cholesterol ester secretion to the cultured medium. In addition, the length of carbon chain bound to cysteine derivative is important for lipid metabolism.

In conclusion, the present study demonstrated for the first time that cycloalliin is a potent inhibitor of apoB100-containing lipoprotein assembly and MTP activity in the liver. In addition, other sulfur-containing amino derivatives such as S-methyl-, S-ethyl-, and S-propyl-cysteines, S-propyl-cysteine sulfoxide, DL-methionine sulfoxide, S-carboxymethyl cysteine, and S-carboxyethyl cysteine exhibit inhibitory effects on the production of apoB100 as well as on the secretion of lipids from the liver. These results suggest that the consumption of these vegetables is favorable for preventing and/or ameliorating certain disease caused by lipid metabolism disorders.

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